XXXV. COLOURING MATTER CONTAINED IN THE SEED-COATS OF *ABRUS PRECATORIUS*.

By SARASI LAL SARKAR.

From the Physiological Laboratory, Medical College, Calcutta.

(*Received May 2nd, 1914*)

The toxic principle contained in the seeds of *Abrus precatorius* has been the subject of elaborate investigations by many workers, including Klein, De Wecker, Sattler, Warden, Waddell etc. But it appears that none has thought it worth his while to investigate the chemical nature or the properties of the colouring matters contained in the coatings of the seeds of *Abrus precatorius*.

Dr D. D. Cunningham, however, has given a description of the microscopical structure of the seeds of *Abrus precatorius* [1882], which I quote below so that the description may be recorded in a journal which is readily available for reference.

"The integument of the seed consists of no less than nine distinct layers of cells differing considerably from one another in their characters.

Proceeding from without inwards we find :—

(1) A layer of considerable thickness but containing only a single stratum of cells. They are thick-walled, columnar cells containing the colouring matter to which the seed owes its bright scarlet hue, and are arranged in radiant fashion around the deeper portions of the integument. Each cell is dilated peripherally and in many cases a slight basal bulbosity is also present. The peripheral dilated portion is cut up into a number of more or less cuneate portions which are closely adapted to one another. In many cases in the mature seeds the central point where the apices of the segment come into relation to one another really consist of a narrow canal leading directly into the cell-cavity. The surface of the seed-coat due to the close apposition of the cells, necessarily comes to present the appearance of being composed of a layer of cuneate facets arranged in rosetted fashion around central points—each rosette representing the extremity of one of the columnar cells, and the central points in many instances being openings, leading directly into the cell-cavities.

(2) A thick stratum of small cells with thick walls and irregular sinuous outlines.

(3) A thick stratum of small, also thin-walled cells.

(4) A thin stratum of small, also thin-walled cells.

(5) A stratum of elongated thin-walled cells.

(6) A stratum of thickened cells two or three layers deep.

(7) A single row of minute thin-walled cells of more or less cubical character.

(8) A stratum of thick-walled cells with dense yellowish granular contents.

(9) A stratum of thickened more or less parenchymatous cells with mere traces of cavities or contents.

The entire thickness of this stratified coating may be broken up in various ways, but apparently the line which may be taken as representing the transition from testa to tegumen runs through the thin-walled cells of the 7th stratum. The cells save in the 1st and 8th stratum appear to be almost or quite devoid of contents."

The above description throws some light on the following peculiar behaviour of these seeds towards certain reagents.

A few entire *Abrus precatorius* seeds are kept in a corked test-tube immersed in chlorine water, along with seeds broken into pieces and with those which have cracks or holes on the surfaces of the testa. The scarlet material contained in the testa of those seeds which are broken or which have cracks or holes in their surfaces is found to be decolourised, while the entire seeds are perfectly unaffected, though some of these were kept in the solution of chlorine water for several months.

When broken *Abrus precatorius* seeds or those which have cracks or holes on their surfaces are kept immersed in a dilute alkaline solution, e.g. of caustic potash or ammonia or simply water, the solutions become coloured owing to the dissolving out of the colouring matter contained in the testa of the seeds, which gradually lose their scarlet hue. The entire seeds without any hole or cracks on their surface, are not in the least affected when kept immersed in these solutions.

The above phenomenon is not due to the presence of any waxy material in the superficial part of the seed-coat, for, if the seeds having the seed-coats entire, i.e. having no cracks or holes in them, are kept immersed in a solution of ether, previously to their treatment with caustic soda solution etc., the seeds are not decolourised as before. The above experiments prove that

(1) there is a structure on the surface of the seeds which is perfectly impermeable to fluids;

(2) the colouring matter which gives a scarlet hue to the seeds is situated beneath this superficial structure;

(3) when any fluid can make its way, at any point, through the superficial impermeable structure this can readily find access to every part of the structure which contains the scarlet colouring matter of the seeds.

These phenomena can be readily understood by taking into account the fact that the superficial layer of cells on the coating of the seeds are not only elongated structures with thick walls, but really consist of two parts, viz. the peripheral portion and the central portion. The peripheral portion is dilated and cut up into cuneate portions, which are so closely applied to one another as to be perfectly impermeable to fluid.

To make the description quoted above consistent with the experiments described we are led to suppose that the openings, which according to Dr Cunningham lead directly into the cell-cavities, are merely blind pouches. In case of an entire seed being kept immersed in a fluid, which has the power of dissolving out the colouring matter contained in the seeds, no colouring matter comes out into the solution because the fluid cannot penetrate through these blind pouches into the portions of the cells which contain the colouring matter.

This property of impermeability of the superficial layer of cells to fluids and its power of thus protecting the internal layers of the seed, is evidently meant by nature for protection of the seeds so that the propagation of the species of the plant may be kept up. It is necessary for the propagation of the species of this plant that the seeds should be able to keep themselves intact for a long time without suffering any disintegration. For the plants die seasonally every year scattering their seeds all round and these germinate fresh plants next year. The above is also in agreement with the views expressed by many observers that the seeds are distributed by being eaten by birds, and passing out unchanged with their excreta. In the *Pharmaceutical Journal*, July 7, 1883, it has been stated that Dr Noura of Brazil, who, while a member of the government commission appointed to regulate the boundary between Brazil and Bolivia, found it in virgin forests where the husbandmen had never penetrated and where the seeds had very probably been carried by birds. C. Owatari in his article on "Botanical Excursion to Formosa (Taiwan)" published in *Botanical Magazine*, XI. Tokyo, 1897, holds however the opposite view. He considers that the attractive colouration of the seed in *Abrus precatorius* might be accounted for as imitating some beetles which are offensive to birds and are thus protected from being devoured." Extended observations are required to decide which

of these views are really correct. I carried on experiments with pigeons, by giving them entire *Abrus precatorius* seeds which they avoided taking while they greedily devoured paddy-grains. So this experimental evidence is in favour of the Japanese observer.

According to Dr Cunningham, as will be seen from the description quoted above, there are two kinds of colouring matter present in the seed-coats. The cells of the 1st layer contain the colouring matter which gives the scarlet hue to the seed-coats. The cells of the 8th layer contain a yellowish colouring matter. I have found by microscopical examination of the section of the seed-coats, that besides the dyes mentioned by Dr Cunningham a kind of black granule is present at the black spot of the seed-coat. This is however insoluble in acids and alkalis and is not a soluble dye but is in the nature of a pigment and so may be omitted from consideration.

For examination of the colouring matters contained in the seed-coats, a quantity of Abrus seeds are taken and the coatings are separated from the cotyledons and are then soaked in water for about four hours and the coloured solution filtered. The filtrate is shaken with ether which extracts traces of yellow colouring matter, which can be identified by the microscope with the yellow colouring matter contained in the 8th layer of the cell. This was obtained in very minute quantities and was not chemically examined any further. The aqueous extract on evaporation leaves a fine pink coloured residue. This is again dissolved in water and copper acetate solution is added in sufficient quantity to cause complete precipitation of the colouring matter. The mixture is left standing for a day and then filtered. The precipitate is repeatedly washed with distilled water, is dissolved in dilute hydrochloric acid, and decomposed by sulphuretted hydrogen. The filtrate from the copper sulphide is then evaporated to dryness in a steam-bath and the residue is extracted with water and filtered. This filtrate contains the colouring matter which gives the scarlet hue to the seed-coats.

The following reactions show that the colouring matter is a tannin substance.

(1) Dilute ferric chloride solution gives a blue black precipitate, the precipitation becoming complete in 24 hours. This reaction with iron salts is not only an evidence that the solution under the examination is a tannin substance, but it also gives indication as to the nature of the chemical composition of this tannin substance. For regarding tannin substances it may be stated as a general truth, that those which give blue-black colourations with ferric salts are derivatives of pyrogallol and gallic acid, while those which afford a green colouration are derived from catechol and protocatechuic acid.

(2) Lead acetate gives a copious blue coloured precipitate.

(3) Allen in his book on commercial analysis says:— "A delicate reaction for tannins (first noticed by the author and apparently general) is the deep red colour produced on treating a solution with potassium ferricyanide mixed with ammonia." When solutions of the colouring matter and of tannic acid are treated side by side with ammoniacal ferricyanide solution for comparison, exactly the same shade of colour is noticed in both cases.

(4) The solution is found to be gradually decolourised by strips of isinglass, which are found to acquire a reddish violet shade. This reaction proves that the reactions of tannin noticed in the solution are not due to any colourless tannin substance, but to the presence of the colouring matter, for it is the colouring matter which is attracted by isinglass.

(5) The colouring matter is precipitated by a solution of gelatin.

(6) On adding stannous chloride solution to the solution, it acquires a pinkish violet shade and after about 12 hours a copious precipitate having the same colour is formed.

(7) The residue obtained by evaporating the solution has an astringent taste like that of tannic acid, and is insoluble in ether, chloroform, benzene and carbon disulphide as is the case with tannins generally.

I should note here that I have carried on the above set of experiments as well as the experiments which will be described below, not only in test-tubes, but where possible by micro-chemical methods also. That is to say I have taken microscopical sections of the seed-coats of *Abrus precatorius*, have treated these with chemical reagents such as solutions of ferric chloride, stannous chloride, lead acetate etc., and have observed under the microscope changes in the colouring matter contained in the 1st layer of cells corresponding with those noticed in the test-tubes. For example, by adding ferric chloride solution in sufficient dilution, we can readily stain this colouring matter violet, leaving other portions of the seed-coats unaffected.

All these experiments, I think, conclusively prove that the colouring matter contained in the 1st layer of the seed-coats of *Abrus precatorius*, is a definite chemical substance of the nature of a tannin.

The reactions of this tannin substance were further studied with the view of finding out whether it can be identified with any known tannin or whether the substance is to be regarded as an altogether new tannin. Some of these reactions are stated below.

(1) Copper sulphate gives a bluish coloured precipitate, which dissolves in ammonia giving a brown coloured solution.

(2) On dissolving crystals of sodium nitrite in the solution on a

porcelain tile, the liquid is coloured from yellowish brown to reddish brown.

(3) With hydrochloric acid a red colour is produced.

(4) Addition of ammonia produces a brown colouration.

(5) Crystals of sodium sulphate added to the solution on a porcelain plate, after some time give yellowish red colour near the edges.

(6) Addition of lime-water causes a bluish coloured precipitate.

(7) Addition of ammonia after addition of ferric chloride solution, causes a violet coloured solution with a precipitate of the same colour.

(8) Tartar emetic solution slowly produces a yellowish brown coloured precipitate.

(9) Tartar emetic and ammonium chloride yield a clay-coloured precipitate.

(10) Nitrous fumes passed into the hot solution, give a yellow colouration and a yellow dye is left on evaporation.

Comparing the reactions stated above, with the reactions of the known tannins one will find that the colouring matter of *Abrus precatorius* seeds appears to be a new tannin substance.

In conclusion I beg to thank the authorities of the Indian Association for the Cultivation of Science for kindly allowing me to work at the laboratory of the Association.

REFERENCES.

Cunningham (1882), *Indian Medical Gazette*, 288.

XXXVI. THE ACTION OF DIAZOMETHANE ON CASEINOGEN (Preliminary Communication).

By ARTHUR GEAKE and MAXIMILIAN NIERENSTEIN.

From the Biochemical Laboratory, Chemical Department, University of Bristol.

(*Received June 4th, 1914*)

Skraup and Krause [1909] methylated caseinogen with methyl iodide and boiling alcoholic potash with the object of showing which of the constituent amino-acids of the protein chain contained free amino- or hydroxyl-groups. Since their product on hydrolysis yielded no tyrosine or lysine and but little histidine and arginine they concluded that such free groups were present in the nuclei of these amino-acids. It is evident that such a vigorous methylation as they employed may be accompanied by an essential hydrolysis of the protein molecule. We have therefore methylated caseinogen with diazomethane in the hope that by this method results of a more conclusive character may be obtained. By the employment of such a mild reagent the possibility of decomposition is reduced to a minimum and it is therefore practically certain that the product obtained is a direct substitution product of caseinogen. By a comparison between the behaviour, on the one hand, of amino-acids and simple polypeptides and, on the other hand, of caseinogen, it is hoped that a direct view may be obtained into the protein molecule as it actually exists. Results have already been obtained which indicate clearly that the presence of carboxyl[1] groups may be proved and their number estimated. It may also be possible to estimate the number of free hydroxyl groups.

It is to be expected that our product, like that of Skraup and Krause, will on hydrolysis fail to give those amino-acids which when combined in the protein chain still contain free groups capable of methylation. It is further hoped that it may be possible to isolate in the form of their methyl

[1] According to T. B. Robertson [1912], for example, the acidity of proteins is not due to carboxyl groups but to groups of the type C(OH)=N.

derivatives amino-acids which in the free state may be unable to resist unchanged the vigorous hydrolysis usually employed.

In the present communication we include only the preliminary investigation of our product. The further investigation of this substance, and the study of the action of diazomethane on amino-acids and polypeptides are both in progress and will form the subject of further communications.

If an ethereal solution of diazomethane is poured over dry caseinogen, a moderate evolution of gas occurs. By the continued action of fresh portions of diazomethane a product is finally obtained which can in this way be no further methylated. The substance thus obtained is indistinguishable in appearance from caseinogen itself, but contains 3·5 % more methyl. In properties it differs most markedly in its insolubility. If a portion is added to an excess of dilute (N/50) caustic alkali a clear solution is obtained only after about 20 hours standing whereas caseinogen similarly treated dissolves in a few minutes. Towards acids methylo-caseinogen is even more stable, being dissolved by dilute sulphuric acid only after several days heating on a water-bath. By the addition of acid to the alkaline solution, a precipitate is formed containing less methyl than the original substance; this is readily re-dissolved by excess of acid.

Nitrogen estimations in our methylo-caseinogen by Kjeldahl's method gave consistently lower results than estimations by Dumas' method. It therefore seemed probable that by boiling with concentrated sulphuric acid part of the nitrogen was evolved as such. All attempts to estimate this nitrogen have however yielded only negative results.

Unlike Skraup and Krause's product our methylo-caseinogen readily gives Millon's reaction and it is therefore doubtful whether the tyrosine nucleus has been methylated. It may be pointed out that the failure of Millon's reaction in the case of their product is sufficiently accounted for by the iodine introduced by the action of methyl iodide, since it is known that iodo-proteins do not give this reaction. The Adamkiewicz-Hopkins reaction is more pronounced with methylo-caseinogen than with caseinogen. Skraup and Krause made the same observation but ascribed it to the presence of iodine.

It was found by Skraup and Krause that caseinogen when heated with hydriodic acid according to the Herzig-Meyer method gives a precipitate of silver iodide. We have been able to confirm this but obtain a lower value (0·36 %) than they (1·13 %) for the percentage of N-methyl; in this we are in agreement with the recent observation of Burn [1914]. Our

methylo-caseinogen contains about the same percentage of methyl (4·36 %) as Skraup and Krause's product (4·26 %).

Experimental Part.

Preparation of Methylo-caseinogen. 500 g. of Kahlbaum's "Casein nach Hammarsten" dried in a vacuum at 40° over phosphorus pentoxide were treated with diazomethane in ethereal solution in portions of about 5 g. The first few additions were followed by a vigorous evolution of gas. After each addition of diazomethane the reaction mixture was allowed to stand in the dark until it became colourless. The methylation was continued for four months and about 75 g. (a large excess) of diazomethane were used. The last few portions of diazomethane were only very slowly decolourised and no evolution of gas was observable. Methyl estimations from time to time by Herzig and Meyer's method showed that a maximum methyl content had been reached. The methylo-caseinogen was filtered from the ethereal mother-liquor and well washed with absolute ether. The ether was then removed by exposure to the air in shallow dishes for several weeks. All smell of ether had disappeared in a few days. For analysis the substance was further purified by extracting in a Soxhlet with absolute ether for a week. Only a minimal amount (less than 0·1 %) of fat was thus removed.

The ethereal filtrate and washings on evaporation yielded about 1 c.c. of a semi-solid mass. This probably consisted of fat or fatty derivatives and was not further investigated.

Methylo-caseinogen thus obtained is a light colourless powder indistinguishable in appearance from caseinogen. As has been mentioned above, it is only dissolved by acids and alkalies with difficulty; by the action of alkali methyl groups are lost. The alkaline solution is optically active.

Analysis. For analysis the pure fat-free methylo-caseinogen was allowed to stand for at least twelve hours exposed freely to the air of the balance room.

Water estimations were made whenever portions were weighed out for analysis. The results were calculated on the weight of water- and ash-free protein. [See Geake, 1914.]

It has been mentioned above that nitrogen estimations by Kjeldahl's method gave consistently lower results than by Dumas' method[1]. It was

[1] Such differences have been previously observed with proteins. Thus Pittom [1914] working with egg albumin obtained a difference between the two methods of more than 1 % nitrogen.

thought probable that this was due to a portion of the diazomethane having reacted with caseinogen to give a product containing diazo-nitrogen. Such a product when heated with concentrated sulphuric acid would give off part of its nitrogen as nitrogen gas. Estimations of diazo-nitrogen by the usual methods of heating with concentrated hydrochloric acid or with somewhat diluted sulphuric acid yielded negative results. A portion of the substance was therefore heated with the usual Kjeldahl mixture (30 g. conc. H_2SO_4, 10 g. K_2SO_4, 2 g. $CuSO_4$), and the gases evolved passed through a heated combustion tube containing copper oxide and a reduced copper spiral. Even in this way only a minimal amount of unabsorbed gas was collected.

The methylo-caseinogen gave the following results on analysis :

0·5086 g. (10·65 % H_2O) ; 0·0041 g. ash
0·5049 g. (10·65 % H_2O) ; 0·0034 g. ash
0·1957 g. (10·04 % H_2O) ; 0·3549 g. CO_2 ; 0·1322 g. H_2O
0·2118 g. (8·72 % H_2O) ; 0·3869 g. CO_2 ; 0·1429 g. H_2O
0·1657 g. (7·60 % H_2O) ; 0·3088 g. CO_2 ; 0·1143 g. H_2O
0·2728 g. (10·07 % H_2O) neutralised 25·89 c.c. N/10 H_2SO_4 (Kjeldahl)
0·2497 g. (10·07 % H_2O) „ 23·24 c.c. „ „
0·2967 g. (8·48 % H_2O) „ 28·58 c.c. „ „
0·2379 g. (8·48 % H_2O) „ 23·08 c.c. „ „
0·3066 g. (10·11 % H_2O) ; 36·1 c.c. N over water at 13·8° and 758 mm.
0·2767 g. (10·04 % H_2O) ; 32·8 c.c. „ „ 12·8° „ 744 mm.
0·2014 g. (8·48 % H_2O) ; 24·8 c.c. „ „ 11·8° „ 745 mm.
0·4998 g. (8·48 % H_2O) ; 0·0264 g. $BaSO_4$
0·4998 g. (8·48 % H_2O) neutralised 30·90 c.c. N/10 NaOH (Phosphorus estn. by Neumann's method.)

$$\text{Ash} = \frac{(0\cdot90)}{(0\cdot76)} = 0\cdot83\ \%$$

100 parts water and ash-free methylo-caseinogen contain

Found					
C	H	N		S	P
		Kjeldahl	*Dumas*		
55·43	7·21	14·90	15·43	0·800	0·755
55·03	7·26	14·62	15·31		
55·46	7·49	14·87	15·73		
		14·97			

Mean composition :

	Methylo-caseinogen	Caseinogen [Geake, 1914]
	%	%
C	55·31	53·20
H	7·32	7·09
N (Kjeldahl) ..	14·84	15·61
(Dumas) ..	15·49	15·63
S	0·800	1·015
P	0·755	0·731

Estimations of methyl were carried out by Herzig and Meyer's method. For convenience all results have been calculated for " CH_3," not for " OCH_3 " or " NCH_3." It was found by Skraup and Krause that caseinogen contains 0·40 % $(O)CH_3$ and 1·13 % $(N)CH_3$. Our results for the latter are considerably lower.

Estimation of methyl in caseinogen:

		AgI			
		HI at 140° C.	HI at 200° C.	HI + NH_4I at 300° C.	
(I)	0·4090 g. (11·99 % H_2O)	0·0134 g.	0·0138 g.	0·0215 g.	
(II)	0·4265 g. (12·00 % H_2O)	0·0166 g.	0·0161 g.	0·0191 g.	
(I)	$(O)CH_3$ = 0·24 + 0·25 = 0·49 %;	$(N)CH_3$ = 0·38 %.	Total CH_3 = 0·87 %		0·88 %
(II)	= 0·28 + 0·27 = 0·55 %;	= 0·33 %	= 0·88 %		

Since the completion of this work Burn [1914] has obtained a similar result (0·97 % total methyl).

No great stress can be laid on these values since, as was observed by Skraup and Krause and confirmed by Burn, amino-acids themselves, though free from methyl groups, give a similar precipitation of silver iodide. We have observed that after heating to 140° C. till no further precipitate can be obtained more silver iodide is formed by heating to 200° C. and more again at higher temperatures. The distinction between $(O)CH_3$ and $(N)CH_3$ cannot therefore be taken as exact.

Skraup and Krause's methylo-caseinogen contained 0·90 % $(O)CH_3$ and 3·36 % $(N)CH_3$, making a total of 4·26 % methyl.

Our methylo-caseinogen gave the following results:

	AgI	
	HI at 140° C.	HI + NH_4I at 300° C.
0·3960 g. (9·74 % H_2O)	0·1110 g.	0·1301 g.
0·3953 g. (9·76 % H_2O)	0·1105 g.	0·1286 g.
0·4340 g. (9·08 % H_2O)	0·1381 g.	0·1494 g.
0·4050 g. (9·08 % H_2O)	0·1210 g.	0·1185 g.

$(O)CH_3$ = 1·99, 1·98, 2·24, 2·10. Mean 2·08 %
$(N)CH_3$ = 2·33, 2·31, 2·42, 2·06 2·28 %
Total methyl = 4·36 %

Since caseinogen gave 0·88 % methyl, the increase of methyl on methylation is 3·48 %.

Colour Reactions. The following reactions are given equally by caseinogen and by our methylo-caseinogen: Biuret reaction, xanthoproteic reaction, lead sulphide reaction, Molisch reaction, Liebermann's reaction, tryptophane reaction with H_2SO_4, vanillin and KNO_2 or with HCl, vanillin and KNO_2, Reichl's reaction and Mörner's test for tyrosine. The Adamkiewicz-Hopkins

reaction and Denigé's test for tyrosine are given more strongly by methylo-caseinogen. The reddish violet colouration observed by Cooper [1913] when proteins are immersed in aqueous quinone solutions is also obtained much more rapidly and powerfully with methylo-caseinogen than with caseinogen.

Since the product gives Millon's reaction and the other tyrosine reactions it is doubtful whether the hydroxyl group of the tyrosine nucleus has been methylated[1]. As mentioned above, the absence of the reaction in the case of Skraup and Krause's product is sufficiently explained by the presence of iodine. Skraup and Krause also observed the more pronounced Adamkiewicz-Hopkins reaction, but ascribed this to the iodine in their substance.

[Note added July 30th. Since this paper was sent to the press we have noticed that Herzig and Landsteiner [1914] have also made experiments on the methylation of caseinogen by diazomethane. As their paper was only published on 27th April we did not see it until some time after the manuscript had left us on June 3rd.]

REFERENCES.

Burn (1914), *Biochem. J.* **8**, 154.
Cooper (1913), *Biochem. J.* **7**, 186.
Geake (1914), *Biochem. J.* **8**, 30.
Herzig and Landsteiner (1914), *Biochem. Zeitsch.* **61**, 458.
Pittom (1914), *Biochem. J.* **8**, 157.
Robertson (1912), *Die physikalische Chemie der Proteine*, Steinkopff, Dresden.
Skraup and Krause (1909), *Monatsh.*, **30**, 447.

[1] Diazomethane has no action on tyrosine in ethereal suspension but in methyl alcohol methylation takes place. Millon's reaction is not given by veratric acid but is given by trimethylgallic acid; the positive reaction is therefore not an absolute proof of the presence of a free hydroxyl group.

XXXVII. THE MECHANISM OF AGGLUTINATION OF BACTERIA BY SPECIFIC SERA.

By WILLIAM JOHN TULLOCH.

From the Bacteriological Laboratory of the University of Durham College of Medicine, Newcastle-upon-Tyne.

(*Received May 15th, 1914.*)

Flocculation of bacteria and other cells by specific antisera, being, if not the simplest, at least the most easily investigated of the "in vitro" phenomena of immunity, offers a suitable field for the study of the mechanism of serum tests in general, for if we knew the factors that control agglutination we might be able to correlate this phenomenon with others—e.g. lysis—which, while apparently very different therefrom, may in reality be closely allied thereto. The immediate purpose of the investigation was, then, to enquire into the factors determining flocculation of sensitised bacteria; while it was hoped that from a study of these an indication for the explanation of biological lysis and other laboratory serum tests on a basis of observed physiological fact might be obtained.

The experiments to be described in the present communication were carried out with a view to the corroboration and amplification of a statement made by Bordet [1899]: "That agglutination depends upon the presence of an electrolyte in the fluid used for suspending the organisms." This author showed that bacteria that have been exposed to the action of specific antiserum, then washed, and suspended in distilled water, form an homogeneous and stable suspension. The stability of this suspension of "sensitised" organisms depends upon the absence of electrolytes from the fluid as is shown by the fact that the addition of a small quantity of NaCl results in complete agglutination of the suspended organisms.

This experiment might be interpreted as indicating that the process of agglutination by antisera is divisible into two phases:

(*a*) A phase in which the antibody of the serum becomes united with its antigen—the organism;

(*b*) A phase in which the united "antibody-antigen couple" is flocculated by the electrolyte of the suspending fluid.

The second phase of the phenomenon is purely physical in character and is comparable with the sedimentation of sand at river estuaries and with the aggregation of particles of "denaturated" protein by the addition of small quantities of electrolytes. It is with the second phase of the reaction that the experiments to be described are mainly concerned.

Before the publication of Bordet's observation [1899] there had been a number of explanations offered of the nature of the reaction of specific agglutination. These were all founded upon chemical bases and failed to explain satisfactorily the clumping of organisms by specific antisera. Thus Gruber and Durham [1896] considered that the serum produced a chemical (?) change in the outer layers of the organismal protoplasm causing them to become sticky. This view of the phenomenon while offering adequate explanation of the mutual adhesion of sensitised bacteria or cells, when they had come into contact with one another, failed altogether to explain why they *should come together to form clumps.*

The next advance made in the investigation and explanation of specific agglutination is due to Krause [1897], who showed that the clear fluid obtained by filtration of an old broth culture of an organism produced a precipitate when mixed with an homologous antiserum. Basing their observations on this fact, Nicolle [1898] and Paltauf [1897] advanced the following hypotheses. The former considered that the phenomenon was really explicable by the theory advanced by Gruber and Durham, but he claimed that that moiety of the organismal protoplasm which was susceptible to the action of the agglutinating serum—"agglutinable substance"—was resident principally in the external layers of the organism and might in old cultures diffuse into the medium. Its precipitation in the organisms themselves led to agglutination while its flocculation from the culture filtrate led to the precipitum observed by Krause. It will be seen that Nicolle's theory involves the acceptance of Gruber's hypothesis and only differs therefrom in correlating the Krause phenomenon with agglutination. Paltauf considers that in all cultures a certain amount of agglutinable substance diffuses into the medium and is precipitated by the addition of antiserum, so forming a coagulum which entangles the suspended bacteria and subsequently contracting leads to the formation of agglutinated masses.

This view of Paltauf's is ingenious, but it presupposes that a coagulum is always formed in the suspending fluid and that this coagulum *is not*

discontinuous. As there is no evidence that these suppositions are valid, the explanation offered must be considered as inadequate. His hypothesis, however, seems to me to be of considerable import in that it draws attention, indirectly at least, to the physical state of the interacting bodies as an important factor in the phenomenon of agglutination.

From the experiments to be described it will be noted that a striking analogy may be drawn between the behaviour of sensitised organisms and that of suspensions of denaturated protein in presence of electrolytes. Furthermore, unsensitised bacteria behave in respect of electrolytes as does fresh protein, and Liefman [1913] has shown that in order to produce flocculation of unsensitised organisms a concentration of 70-80 % $(NH_4)_2SO_4$ is required. With different organisms different concentrations of the salt appear to give optimum results, therein exhibiting a striking resemblance to the fractional salting out of proteins from solution by similar reagents. Agglutination of sensitised bacteria on the contrary occurs in presence of, and is conditioned by low concentrations of, salts, e.g. physiological saline, and has, I think, its analogue in the aggregation of denaturated protein particles by low concentrations of salts. This subject of the aggregation of denaturated protein by the addition of salts has been fully investigated by Chick and Martin [1912], who show that flocculation under these circumstances depends upon the neutralisation of the surface electric charge carried, according to the Lippman-Helmholtz hypothesis, by particles or molecules in suspension.

If the hypothesis, then, "that aggregation of denaturated protein particles and agglutination of sensitised bacteria are similar phenomena" be correct, we should expect sensitised bacteria to exhibit certain of the features characteristic of denaturated protein. In respect of electrolytes, particles of denaturated protein recall, in certain respects, in their reactions those of rigid colloids; thus the charge carried by such colloids is largely determined by the reaction of the suspending fluid, and the valency of the ions of the electrolyte in which the colloid is suspended is an important factor in its precipitation. We should expect then, if our hypothesis be correct, that the presence of hydrion and hydroxidion would markedly affect the process of agglutination and that in certain experimental conditions the valency of the kation of the electrolyte would determine the aggregation of the organisms while in other circumstances the clumping would be conditioned by the valency of the anion.

Experiments to show that Variation of the Intrinsic Pressure and Viscosity of the suspending fluid has no marked influence on the Reaction.

As the disposition of particles suspended in a fluid is largely determined by the electric charge carried by the particles and by the intrinsic pressure of the suspending fluid, one can alter the disposition of the particles by interfering with either of these factors. These are not infrequently interdependent, but without altering the intrinsic pressure of a fluid we may bring about flocculation of particles suspended in it by neutralisation of the surface charge carried by the particles, and I conceive of agglutination as being dependent on this mechanism. It seemed possible, however, that flocculation of organisms by antisera might depend upon alteration of the intrinsic pressure of the suspending fluid, and with a view to elucidating this point, agglutination reactions were carried out in presence of 0·9 % NaCl, 0·9 % NaCl dissolved in 10 % alcohol, and 0·9 % NaCl dissolved in 1 % gelatin.

Examination of these fluids by means of Traube's stalagmometer showed that for a given volume of the fluids at 37°—the temperature at which the reactions were carried out—

0·9 % NaCl equalled 58·5 " standard " drops,
0·9 % NaCl in 10 % alcohol = 89·5 drops,
0·9 % NaCl in 1 % gelatin = 65·2 drops,

thus indicating a very considerable difference in the intrinsic pressure of the solution in alcohol as compared with the others.

Employing the viscosimeter and working at the same temperature, it was found that for a given volume to pass through the tube

0·9 % NaCl required 2 min. 15 secs.,
0·9 % NaCl in 10 % alcohol required 3 min. 20 secs.,
0·9 % NaCl in 1 % gelatin required 3 min. 15 secs.,

thus showing a marked increase in the internal friction in both the alcoholic solution and the gelatin solution.

The following agglutination reaction was carried out, using the above fluids for diluting the serum and for suspending the organisms. Throughout the experiments the organism used was *B. paratyphosus* " *b.*"

TABLE I. 2 *hours at* 37°.

Fluid employed	Dilutions of agglutinating serum						
	1/100	1/500	1/1000	1/2000	1/4000	1/8000	1/16000
0·9 % NaCl	+	+	+	+	+	+	–
„ in 10 % alcohol..	+	+	+	+	+	–	–
„ in 1 % gelatin ..	+	+	+	+	+	+	–

Note. In this and the following experiments + indicates a positive result.

From this it is seen that notwithstanding considerable variation in the intrinsic pressure and internal friction of the fluids in which the reaction was carried out, the result is not markedly different in the case of any of the fluids.

Flocculation would appear then to be due to a direct interaction of "antibody-antigen" complex with electrolyte, and, within the limits of the experiment at least, to be independent of the intrinsic pressure and viscosity of the suspending fluid. This agrees with the findings of Gengou [1908], who states that the flocking out of red corpuscles from suspension by the addition of colloids is due "fundamentally to a direct interaction of the two bodies," *i.e.* colloid and cell.

It was shown by the same author that alteration of the physical state of the interacting bodies leads on the contrary to very different results. Thus if a suspension of $BaSO_4$ be added to fresh serum, the $BaSO_4$ is held in suspension, but its addition to the same serum previously heated to 65° results in sedimentation.

According to Gengou's views, when the $BaSO_4$ is added to fresh serum it remains dispersed because, adhering to the colloidal particles of the serum which show no tendency to spontaneous flocculation, the particles of $BaSO_4$ are kept apart from one another. Added to heated serum or to red blood cells the $BaSO_4$ does rapidly sediment, for both these colloids tend to flocculate of their own accord and naturally do so more rapidly when they have adsorbed the heavy $BaSO_4$.

I quote these experiments in order to emphasise the important influence that the physical state of the reacting bodies has upon the phenomena of immunity reactions and to call attention to the points that require special consideration in the investigation of these, particularly in respect of agglutination.

INFLUENCE OF THE ELECTROLYTE UPON THE TITRE OF THE SERUM.

In view of the findings of the above experiments, I decided to proceed with the investigation of the reaction from the standpoint of its probable analogy with the aggregation of denaturated protein particles.

In the first series of experiments, agglutination tests were performed in presence of a variety of electrolytes in order to determine whether any of the electrolytes employed affected the titre of the agglutinating serum.

Technique. A 24 hours agar culture of the organism (*B. paratyphosus* "*b*") was washed off in distilled water and centrifuged, the supernatant fluid was pipetted off and to the deposit was added distilled water, the mixture well shaken and again centrifuged. This process was repeated twice and the deposit obtained after the last centrifuging was triturated and suspended in distilled water. To ensure the homogeneity of this suspension it was filtered through coarse filter paper.

This distilled water suspension was exposed to the action of varying dilutions of serum in presence of different electrolytes, the concentration of which was so arranged that with the addition of the distilled water suspension to each tube the electrolytes were equimolecular with 0·9 % NaCl. Employing this technique the following result was obtained:

TABLE II. 2 *hours at* 37°.

	Dilutions of Serum						
	1/100	1/500	1/1000	1/2000	1/4000	1/8000	1/16000
NaCl ..	+	+	+	+	+	+	–
NaF ..	+	+	+	+	+	+	–
Na_2SO_4 ..	+	+	+	+	+	+	–
K_2SO_4 ..	+	+	+	+	+	+	–
Na_2CO_3 ..	–	–	–	–	–	–	–
Na_2HPO_4	+	+	+	+	+	+	–
$BaCl_2$ ..	+	+	+	+	+	+	–
$Na_3Cit.$ ..	+	+	+	+	+	+	–
Aq. Dest.	+	–	–	–	–	–	–

Note. The positive result obtained with 1/100 dilution of the serum in water is probably due to the salts that the serum contains.

In the above reaction the concentrations of serum used were chosen with a view to showing whether any of the salts employed exercised a *marked* influence upon the titre of the serum.

From the above table the following points are made clear:

1. That for all the salts employed, in the above experimental conditions, the titre of the serum remains the same, provided that the solutions of the salts give an approximately neutral reaction.

2. That the clumping of sensitised organisms is independent of the presence of the Ca ion. If it were dependent on the presence of the Ca ion, agglutination would probably not occur in presence of fluoride.

I draw attention to this on account of the important function that Ca subserves in many vital phenomena.

3. That salts which hydrolyse to produce OH ions have not the property of agglutinating bacteria in presence of specific agglutinating serum.

This point is shown more strikingly in the following table in which are compared the actions of " acid " and " basic " carbonates and phosphates of sodium.

TABLE III. 2 *hours at* 37°.

	Dilutions of Serum						
	1/100	1/500	1/1000	1/2000	1/4000	1/8000	1/16000
NaCl ..	+	+	+	+	+	+	−
Na_2CO_3 ..	−	−	−	−	−	−	−
$NaHCO_3$ ..	+	+	+	+	+	−	−
Na_3PO_4 ..	−	−	−	−	−	−	−
Na_2HPO_4	+	+	+	+	+	+	−

Experiments on the Inhibition of Agglutination by Alkalis.

This inhibitory action of alkalis upon agglutination is of considerable interest in view of the influence that the reaction of the suspending fluid has upon the aggregation of particles of " denaturated " protein [Chick and Martin 1912; Hardy 1899], and the following series of tests was set up with a view further to elucidate the conditions that determined the inhibitory action of alkalis upon the flocculation of sensitised bacteria.

It might well be that the inhibition is essentially due to the same cause in each case and that this is probable is shown from the following results.

Technique. A 24 hours agar culture was washed off in saline and agglutinated at 22° in presence of 1/1000 agglutinating serum (titre—1/8000). The supernatant fluid was pipetted off and the material was washed twice with distilled water as in the previous series, then triturated, filtered and exposed to the action of varying dilutions of NaOH in presence of the following electrolytes each of which was, when all the reagents were added, equimolecular with 0·9 % NaCl.

TABLE IV. 2 *hours at* 37°.

	No NaOH Control	NaOH N/100. 1 cc.	0·9 cc.	0·8 cc.	0·7 cc.	0·6 cc.	0·5 cc.	0·4 cc.	0·3 cc.	0·2 cc.	0·1 cc.
NaCl ..	+	−	−	−	−	−	−	−	−	−	+
K_2SO_4 ..	+	−	−	−	−	−	−	−	−	−	+
KI ..	+	−	−	−	−	−	−	−	−	−	−
$NaHCO_3$ ..	+	+	+	+	+	+	+	+	+	+	+
Na_2HPO_4	+	+	+	+	+	+	+	+	+	+	+

This shows that until the OH ion is present in the suspending fluid in a concentration equal to that in N/500 NaOH it fails to inhibit agglutination. This concentration is presumably not reached in the case of $NaHCO_3$ and Na_2HPO_4 in the above experimental conditions, for the addition of the NaOH to the solutions leads probably to an increase in the quantity of ionised Na_2CO_3 and Na_3PO_4 in the solutions of these "acid" salts, this being necessarily accompanied by a removal from the fluid of OH ions available for the inhibitory action.

As this experiment showed that salts having a "replaceable" H group in the anion behaved in a manner different from other salts in respect of this alkali inhibition, it appeared of interest to show that salts containing an "irreplaceable" H group in the anion did not exhibit the same peculiarity.

Technique. A 24 hours agar culture was washed off with distilled water, then washed and centrifuged twice. The deposit obtained was suspended in distilled water and added to tubes containing serum and electrolyte of such concentration that with the addition of the emulsion the following dilutions of serum were obtained, the electrolytes being equal in concentration to 0·9 % NaCl.

TABLE V. 2 *hours at* 37°.

	Dilutions of Serum 1/100	1/500	1/1000	1/2000	1/4000	1/8000	1/16000
NaCl	+	+	+	+	+	+	−
NaCl in N/100 NaOH	−	−	−	−	−	−	−
CH_3COONa	+	+	+	+	+	+	−
CH_3COONa „ „	−	−	−	−	−	−	−
CCl_3COONa	+	+	+	+	+	+	−
CCl_3COONa „ „	−	−	−	−	−	−	−
$NaHCO_3$	+	+	+	+	+	+	−
$NaHCO_3$ „ „	+	+	+	+	+	−	−

The inhibition of agglutination by the hydroxidion is probably akin to the prevention of aggregation of protein particles in presence of an alkali first noted by Hardy and interpreted by him as a phenomenon depending

upon disturbance of the electrostatic condition of the particles in suspension. For optimum aggregation the reaction must occur in a solution that is approximately neutral, for protein particles acquire a positive charge when suspended in acid fluids and a negative charge when suspended in alkaline fluids.

To obtain the best conditions for precipitation, this charge must be neutralised, *i.e.* the solution must be so adjusted that it is " isoelectric " with the particles.

It appears probable then that the agglutination of sensitised bacteria also depends on their being suspended in an " isoelectric " fluid.

In what way the alkali produces the inhibition of clumping is by no means clear, for it might obviously act upon the serum, the organism, the union of the serum and organism, or upon the actual flocculation.

From what has been said above, the last of these is seemingly the most rational explanation of the dispersion.

Paal and Voss [1904] suggest that the inhibition of coagulation (aggregation) of protein by hydroxidion depends upon the alkali acting upon the protein and producing a degree of hydrolysis that results in the production of a layer of protective colloid around each particle in suspension. If such a colloid is produced by the action of the OH ion on bacterial protoplasm, its properties disappear when the organisms exposed to the alkali are well washed in distilled water as is shown by the following experiment.

Technique. A 24 hours agar culture of organisms was washed off with N/100 NaOH and exposed to this reagent for 2 hours at 37°. The suspension was then centrifuged, the supernatant fluid pipetted off and replaced by distilled water. The mixture was well shaken and again centrifuged. This washing process was repeated twice and the organisms were then exposed to the following dilutions of an agglutinating serum.

TABLE VI. 2 *hours at* 37°. *Electrolyte used,* 0·9 % NaCl.

	Dilutions of Serum						
	1/100	1/500	1/1000	1/2000	1/4000	1/8000	1/16000
Untreated organisms	+	+	+	+	+	?	–
Treated as above	+	+	+	+	+	?	–

From this it is seen that organisms that have been exposed to the action of alkali and then washed react in the same way as do organisms that have not been so treated. This shows that the OH ion has no permanent effect upon the protoplasm of the bacteria.

Further, that the alkali has no permanent effect on the serum is shown in the following experiment.

Technique. Serum was diluted 1/50 in N/100 NaOH in 0·9 % NaCl. This mixture was placed in the incubator at 37° for 2 hours and was then diluted with 0·9 % NaCl to obtain the following dilutions of serum when the suspension of organisms—in saline—was added to the tubes. In this way both serum and alkali were being diluted in the series.

TABLE VII. 2 *hours at* 37°.

	Dilutions of Serum						
	1/100	1/500	1/1000	1/2000	1/4000	1/8000	16000
Serum alone ..	+	+	+	+	+	?	–
Serum plus alkali	–	–	+	+	+	–	–

As is seen, the agglutination is negative in the tubes containing the higher concentrations of serum plus the higher concentration of alkali, the results become positive with dilution of the reagents. It is apparent then that the alkali has no permanent effect on the serum.

The inhibition of clumping in alkaline suspension is due then to the presence of the OH ion and does not depend upon any permanent effect that the alkali has upon either the organisms or the serum.

The probability is that the inhibition is dependent upon alteration of the electrostatic condition of the suspended organisms, and is therefore the exact counterpart of the dispersion of denaturated protein particles exposed to similar reagents. It is of interest to note in this connection that Chick and Martin [1912] failed to show evidence of hydrolysis of pure protein heated to 100° for 2 hours in presence of sufficient alkali to prevent coagulation[1].

[1] This influence of the OH ion may possibly account for certain cases of "negative phase" that occur in high concentrations of an agglutinating serum. Thus, one not infrequently finds (percentage = 6) that a typhoid serum that fails to give a positive Grünbaum-Widal reaction in a dilution of 1/30 may bring about agglutination in a dilution of 1/500 of the serum.

I merely offer this as a remotely possible explanation of certain of these negative phases, for phosphates with replaceable H groups are found in serum and as in presence of these a fair quantity of alkali is required to give the OH ion concentration necessary to produce inhibition, such an explanation is open to severe criticism, but the phosphates are present in small quantity only and constitute about 0·1 % of plasma by weight and as the alkalinity of the sera might be increased by contact with the soft glass of the capillary tubes in which the blood is collected, this explanation of the negative phase is quite possibly correct in a certain percentage of cases.

EXPERIMENTS ON THE INFLUENCE OF THE HYDROGEN ION ON AGGLUTINATION.

As the presence of hydroxidion has so marked an effect upon the phenomenon of clumping, we should expect, if the hypothesis that the inhibition is dependent upon alteration of the electrical state was correct, that hydrion would also induce an inhibition of agglutination.

That deviation from the isoelectric point leads also to inhibition if such deviation be on the acid side as well as if it be on the alkaline side is shown by the following experiments.

Technique. The procedure was much the same as that used in the previous series (Table IV), except that the concentrations of reagents employed were slightly modified. The organisms were sensitised with 1/100 serum, then washed twice, triturated, and agglutinated in presence of N/20 of the following electrolytes, varying proportions of N/50 HCl being added to each tube as shown in the following table.

TABLE VIII. 2 *hours at* 37°.

Electrolyte N/20	1 cc.	0·9 cc.	0·8 cc.	0·7 cc.	0·6 cc.	0·5 cc.	0·4 cc.	0·3 cc.	0·2 cc.	0·1 cc. N/50 HCl
NaCl	–	–	–	–	–	–	–	–	–	–
Na_2SO_4.. ..	–	–	–	–	–	–	–	–	+	+
$Na_3Cit.$ ..	+	+	+	+	+	+	+	+	+	+
$BaCl_2$	–	–	–	–	–	–	–	–	–	–
Ce_2Cl_6 N/2000	–	–	–	–	–	–	–	–	–	–

These results, which are the converse of those obtained in the corresponding experiment in which alkali was used to inhibit agglutination (Table IV), show the following striking phenomenon. The result obtained with Na_2SO_4 differs from that obtained with NaCl in that a greater concentration of the H ion is required to inhibit agglutination when the former salt is used as the precipitating electrolyte than when the latter is so employed. As both these salts are neutral, there is obviously some other factor than mere concentration of hydrion that must be taken into consideration. This point will be considered in dealing with the relation of the valency of the anion to the flocculation of sensitised bacteria suspended in acid fluids. Attention, however, may be drawn here to the absence of inhibition when citrate is employed which is probably due to two factors: (1) neutralisation of the acid, and (2) the valency of the anion.

That the effect of the acid is not dependent upon any permanent action upon the protoplasm of the organism was demonstrated as follows:

Technique. A saline suspension of the organism was exposed to the action of N/50 HCl for 2 hours at 37°, then centrifuged and washed twice and exposed to the action of serum in saline in the following dilutions.

TABLE IX. 2 *hours at* 37°.

	Dilutions of Serum						
	1/100	1/500	1/1000	1/2000	1/4000	1/8000	1/16000
Untreated organisms	+	+	+	+	+	–	–
Treated as described	+	+	+	+	+	–	–

That the acid has no permanent effect on the serum is apparent from the following experiment.

Technique. Serum in a dilution of 1/25 was exposed to the action of N/25 HCl in saline. This was then diluted (with saline) to produce the following concentrations of serum and acid with the addition of the bacillary emulsion to each tube.

TABLE X. 2 *hours at* 37°.

	Dilutions of Reagents						
	1/100	1/200	1/400	1/800	1/1600	1/3200	1/6400
Serum alone	+	+	+	+	+	+	–
Serum plus acid	–	–	–	+	+	+	–

Note. If the above mixtures are left in the incubator longer than 2 hours there is decided sedimentation in all the tubes, the precipitum however differs from true agglutination in that the flocculi are very small while the supernatant fluid remains markedly turbid.

From the experiments in the two preceding sections, the following conclusions may be drawn:

1. Agglutination is markedly affected by the reaction of the suspending fluid, and deviation—but with certain limits—from the "isoelectric point" results in inhibition of agglutination.

2. The inhibition is conditioned by the presence of hydrion and hydroxidion and does not result from permanent change produced in the protoplasm of the organism or the character of the serum by the action of such ions.

3. Agglutination probably depends upon the neutralisation of charge carried by particles in suspension.

Experiments on the Influence of the Valency of Kation and Anion.

As previous experiments failed to show any variation in the activity—*i.e.* "titre"—of serum in presence of a variety of electrolytes whose solutions were approximately neutral and equimolecular with 0·9 % NaCl, the following experiment was carried out with a view to investigating the relative activity of a number of electrolytes acting upon sensitised organisms.

Technique. A 24 hours agar culture was washed off by saline and agglutinated at 22° for 18 hours in presence of 1/1000 agglutinating serum

(titre—1/8000). The supernatant fluid was pipetted off and the material was centrifuged and washed twice, then triturated and exposed to the action of the following salts.

TABLE XI. 2 *hours at* 37°.

	Dilution of Electrolytes					
	0·5 N	0·1 N	0·01 N	0·001 N	0·0001 N	0·00001 N
NaCl ..	+	+	+	−	−	−
Na_2SO_4 ..	+	+	+	−	−	−
$Na_3Cit.$ ..	+	+	+	−	−	−
$BaCl_2$ ..	+	+	+	+	−	−
$LaCl_3$ ..	0	0	0	+	+	−

0 = not done.

From these results it would seem that the valency of the kation of the electrolyte is the most important factor in conditioning the agglutination of sensitised organisms in the above experimental conditions.

Lest however the result obtained might have been due to a specific action of the salts employed, the following experiment with the salts of the alkaline earths was carried out.

TABLE XII. 2 *hours at* 37°.

	Dilution of Electrolytes														
	0·005	0·004	0·003	0·002	0·001	0·0009	0·0008	0·0007	0·0006	0·0005	0·0004	0·0003	0·0002	0·0001	Normal
NaCl ..	+	+	+	−	−	−	−	−	−	−	−	−	−	−	
KCl ..	+	+	+	−	−	−	−	−	−	−	−	−	−	−	
$CaCl_2$ ..	+	+	+	+	+	+	+	+	+	+	+	−	−	−	
$SrCl_2$ ..	+	+	+	+	+	+	+	+	+	+	+	−	−	−	
$BaCl_2$ ..	+	+	+	+	+	+	+	+	+	+	+	−	−	−	
$MgCl_2$ ..	+	+	+	+	+	+	+	+	+	+	+	−	−	−	

This experiment shows that the result obtained cannot be explained on the hypothesis that it was due to any specificity of action of the salts employed.

These experiments agree with the findings of Coplans [1913] and of Beckhold [1904] that the agglutinating activity of an electrolyte depends upon the valency of its kation. The phenomenon is comparable too with the observations of Chick and Martin [1912] on the aggregation of particles of denaturated egg albumin. These authors show that egg protein dispersed by the addition of alkali to the suspending fluid is more susceptible to the action of salts with divalent kations than to those with monovalent kations while trivalent kations are more active than are divalent.

This, however, is not true of organisms that are suspended in acid fluids

and if agglutination be really comparable to the aggregation of denaturated protein one would not expect it to be so.

Moreover, if the process of sensitisation be akin to denaturation the anion should, in the case of acid suspensions, be the precipitating ion. To determine the validity of this hypothesis, the following experiment was carried out.

Technique. Organisms were agglutinated in saline for 2 hours at 37° in presence of 1/100 serum. These were then centrifuged and washed once in distilled water and then suspended in N/500 HCl. This suspension was added to equal volumes of the electrolytes so that the concentration of HCl in each tube was equal to N/1000, and the concentrations of the salts were such that with the addition of the suspension the following concentrations were obtained.

TABLE XIII. 2 *hours at* 37°.

	Dilutions					
	N/20	N/100	N/200	N/400	N/800	N/1600
NaCl	–	–	–	–	–	–
Na_2SO_4 ..	+	+	+	+	+	+
$Na_3Cit.$ ..	–	–	–	+	+	+
$BaCl_2$ ..	–	–	–	–	–	–
Ce_2Cl_3 ..	–	?	+	+	+	+

It will be seen that the result obtained is only in partial agreement with what one would expect, and the action of two of the salts requires special note.

(*a*) In the case of the citrate the absence of agglutination in presence of the higher concentration of the salt is probably due to the trivalent citrate ion conferring a strong negative charge upon the bacteria and so causing their dispersion.

That polyvalent ions may have such an effect is clear from the work of Burton [1906] on the influence of electrolytes upon the direction of movement of colloid particles exposed to the action of salts in an electric field. He found that the addition of aluminium ion to hydrosols of gold and silver in certain concentrations leads to the precipitation of the metal from its sol ; lower concentration of the salts of aluminium did not have this effect and the particles, which in the sols of these metals bear a negative charge, continued to move in the same direction as before but at a diminished rate. If a concentration of aluminium ion greater than that required to bring about precipitation were employed, the particles remained dispersed, but

now their direction of movement in the field was reversed, indicating a reversal of charge carried by the particles of the sol.

In the case of copper sols, which carry a positive charge, exactly the opposite was noted—polyvalent anions in this case leading to the reversal of direction.

In the experiment described the effect of the citrate is apparently that of a polyvalent anion on a suspension bearing a negative charge. A positively charged and therefore dispersed protein (protein in acid suspension carries a positive charge) is precipitated in presence of certain concentrations of a salt with a trivalent anion, but with greater concentration of the same salt, owing to an overplus of negative charge being conferred upon the particles, they again become dispersed.

(*b*) The other point of special interest is the occurrence of a positive result with the cerium salt in acid suspension. I cannot suggest an adequate explanation of this peculiar finding but it will be seen from further experiments that salts with polyvalent kations behave in a manner very different from salts with mono- and di-valent kations in respect of their precipitation of bacterial suspensions.

In acid suspension as in alkaline suspension, then, sensitised bacteria behave as do denaturated protein particles and the results obtained agree in principle at least with those obtained by the authors above quoted [Hardy 1899; Chick and Martin 1912].

This finding further correlates the two phenomena, viz. aggregation of denaturated protein and agglutination of sensitised bacteria, so adding weight to my thesis that the process of sensitisation is similar to that of "denaturation."

Further Investigation of the Influence of the Valency of the Kation.

The comparative action of Na, Ba, and La ions was next examined in further detail to determine if possible the quantitative relation existing between these ions in respect of their power of producing agglutination.

Technique. The organisms were sensitised as in the previous experiments and the concentrations of the salts were such that with the addition of the suspension the following concentrations were obtained.

TABLE XIV. 2 *hours at* 37°.

	·1	·01	·005	·004	·003	·002	·001	·0009	·0008	·0007	·0006	·0005	·0004
					Sensitised Organisms								
NaCl	+	+	+	+	?	−	−	−	−	−	−	−	−
$BaCl_2$	+	+	+	+	+	+	+	+	+	+	+	+	+
$LaCl_3$	0	0	+	+	+	+	+	+	+	+	+	+	+
					Unsensitised Organisms								
NaCl	−	−	−	−	−	−	−	−	−	−	−	−	−
$BaCl_2$	−	−	−	−	−	−	−	−	−	−	−	−	−
$LaCl_3$	0	0	?	+	+	+	+	+	+	+	+	+	+

	·0003	·0002	·0001	·00009	·00008	·00007	·00006	·00005	·00004	·00003	·00002	·00001	Normal
						Sensitised Organisms							
NaCl	−	−	−	−	−	−	−	−	−	−	−	−	
$BaCl_2$	?	−	−	−	−	−	−	−	−	−	−	−	
$LaCl_3$	+	+	+	+	+	+	+	+	+	+	−	−	
						Unsensitised Organisms							
NaCl	−	−	−	−	−	−	−	−	−	−	−	−	
$BaCl_2$	−	−	−	−	−	−	−	−	−	−	−	−	
$LaCl_3$	+	+	+	+	+	+	+	+	+	+	−	−	

0 = not done.

This result is interesting for it bears some resemblance to the formula

$$1 : X : X^2$$

suggested by Linder and Picton [1895] for expressing the relative precipitating activity of mono-, di- and tri-valent kations acting upon colloidal As_2S_3. The particles of this colloid, like organisms suspended in saline without previous treatment, carry a negative charge. The relative values of the kations as precipitating ions in the experiments of these investigators using the above colloid were

$$1 : 35 : 1023,$$

while in the above experiment taking the value for NaCl (0·003) as unity, the values of barium and lanthanum being 0·0003 and 0·00002 respectively, we obtain approximately

$$1 : 10 : 100.$$

Although this result is of considerable interest, I hesitate to lay any stress upon it, for the action of the salts with polyvalent kations is so different from that of other salts that they are not really comparable.

Investigation of the Action of Polyvalent Kations.

Gengou in an article on molecular adhesion [1908] points out that bacteria which have been exposed to the action of aluminium salts and washed in distilled water are agglutinated on the addition of NaCl. He says, " We

have also found that bacteria that have been agglutinated by moderate doses of alum and then washed in distilled water are well agglutinated on the addition of NaCl (this fact may be compared with the agglutination of bacteria by specific sera and with the observations of Beckhold [1904] on the flocculation of bacteria by the salts of heavy metals). If we treat bacteria that have been mixed with alum in a citrated medium in the same way, the subsequent agglutination by NaCl fails to occur. It is evident then that the mode of action of the citrate in this instance seems comparable to its action on substances in aqueous suspensions (barium sulphate, calcium fluoride, or mastic); but, owing to the fact that chemical reactions may take place between alum and citrate, and thus prevent the manifestations produced by the alum, we think it better to reserve any interpretation of this fact."

As Beckhold [1904] and other observers also call attention to similar phenomena, it seemed of interest to examine the action of salts with polyvalent kations on sensitised and unsensitised organisms.

Technique. (*a*) For the preparation of sensitised organisms, the growth was washed off into saline and exposed to the action of 1/500 serum (titre—1/8000) at 22° for 18 hours. The suspension was then centrifuged and washed with distilled water twice, then suspended in water and exposed to the action of the following electrolytes.

(*b*) For preparation of unsensitised organisms, the 24 hours growth was washed off with distilled water, then allowed to stand and centrifuged to obtain a deposit of organisms. The deposit was washed twice and a suspension in distilled water made as before.

Exposing these to the action of the following salts for 2 hours at 37°, agglutination took place thus:

TABLE XV. 2 *hours at* 37°.

		N/20	N/40	N/100	N/200	N/500	N/1000	N/2000	N/5000	N/10000	N/20000	N/40000
		Sensitised Bacilli										
$NaCl$	..	+	+	+	+	−	−	−	−	−	−	0
$BaCl_2$	..	+	+	+	+	+	+	+	−	−	−	0
$LaCl_3$	..	0	0	0	+	+	+	+	+	?	−	0
Ce_2Cl_5	..	0	0	0	+	+	+	+	+	+	−	0
$AlCl_3$	..	−	−	−	−	−	+	+	+	+	−	−
		Unsensitised Bacilli										
$NaCl$	..	−	−	−	−	−	−	−	−	−	−	−
$BaCl_2$	..	−	−	−	−	−	−	−	−	−	−	−
$LaCl_3$	..	0	0	0	p	+	+	+	+	−	−	−
Ce_2Cl_5	..	0	0	0	sl	sl	p	+	+	+	sl	0
$AlCl_3$	..	−	−	−	−	−	−	+	+	+	+	−

0 = not done p = partial sl = very slight

The salts with polyvalent kations, then, behave in a manner very different from the salts of mono- and di-valent kations—for they are apparently as active in agglutinating unsensitised organisms as in agglutinating sensitised organisms.

I hesitate to offer an explanation of this phenomenon, but it probably depends partly at least on the alteration of the sol form into the gel form of the hydroxides, as pointed out by Coplans [1913], for it is possible that the suspension of bacteria when prepared in the above manner has a slightly alkaline reaction, and this causing the formation of a gel form of the hydroxide gives rise to clumping by entanglement of the organisms in the contracting gel.

This would explain, in part at least, the negative phase obtained with the higher concentrations of $AlCl_3$. The solution of this salt is acid owing to its hydrolytic decomposition, and the physical state of the hydroxide formed by adding the suspension will vary with the dilution of the salt, and as the agglutination by aluminium salts in all concentrations is inhibited by the presence of acid in the suspending fluid, this factor must be considered as a possible cause of the negative phase in the above experiment.

But the negative phase occurring with the higher concentrations of a polyvalent kation may be explained otherwise. It is possible that the polyvalent kations may produce a reversal of charge carried by organisms in the same way as it caused a reversal of direction of movement of the particles of metal sols of gold and silver in the experiments of Burton.

In order further to investigate this point $TaCl_5$ was made use of. This salt is very sparingly soluble in water and does not undergo hydrolysis with any concentration of the salt, therefore the addition of organism suspension should lead to the production of a hydroxide in the same physical state in each case, unless the concentration of the salt itself has a marked influence on the physical state of the hydroxide formed.

Employing the saturated solution of the salt, the following result was obtained.

TABLE XVI. 2 *hours at* 37°.

	Sensitised Organisms						
$TaCl_5$ Sat. ..	1 cc	0·5 cc.	0·2 cc.	0·1 cc.	0·05 cc.	0·02 cc.	0·01 cc.
	−	−	+	+	−	−	−
	Unsensitised Organisms						
$TaCl_5$ Sat. ..	−	p	+	+	−	−	−

This result shows that the negative phase in the case of polyvalent kations is not *wholly* explicable on the grounds that it is due to acid inhibition and it seems improbable—though I make the suggestion with reservation—that it is due in the case of tantalum to the formation of a hydroxide in a special state determined by the conditions of the experiment. In this connection the experiments of Gengou are of importance. The results obtained by that author suggest that, notwithstanding the fact that with $TaCl_5$ a negative phase does occur, the precipitating action of the polyvalent kations is partly explicable on the hypothesis that it is due to the formation of a hydroxide in accordance with the views of Gengou [1908].

The dispersion with the higher concentrations of the polyvalent kations may however be due to a reversal of charge produced by these reagents apart from that due to hydrolytic decomposition of the salts used.

In order to obtain further information on this point I decided to carry out the following experiment.

Technique. A large culture of the organism was washed off with saline and the suspension was divided into two parts, "*a*" and "*b*," each of 5 cc. volume. To "*a*" was added agglutinating serum 1/500, while "*b*" was left unsensitised. After 18 hours at 22° the liquids were centrifuged, the deposit washed in distilled water, again centrifuged and the supernatant fluid decanted and replaced by N/250 $AlCl_3$. Both were then allowed to interact for 2 hours at 37°. The liquids were again centrifuged and the deposit washed as before, and suspensions of the organisms were made in distilled water.

These suspensions were mixed (1) with NaCl, and (2) with NaCl in presence of N/200,000 $Na_3Cit.$, so that the concentrations shown in the following table were obtained in the tubes, when all the reagents were mixed.

Agglutination was allowed to proceed for 2 hours at 37°. The following results were obtained.

TABLE XVII. 2 *hours at* 37°.

	N/20	N/100	N/200	N/400	N/800	N/1600	NaCl
Sensitised Organisms							
NaCl	+	+	+	+	+	+	
NaCl plus citrate ..	−	−	−	−	−	−	
Control	−	−	−	−	−	−	
Unsensitised Organisms							
NaCl	+	+	+	+	+	+	
NaCl plus citrate ..	−	−	−	−	−	−	
Control	−	−	−	−	−	−	

Whether the polyvalent kations have a double action on the organisms is not clear. It might be that they act both as denaturating agents and as precipitating agents, and if this were so the agglutination by the polyvalent kations would be comparable to specific agglutination by sera in presence of mono- and di-valent ions.

If they do not act in this dual capacity, it is highly probable that the sedimentation is dependent on the formation of hydroxides.

I had hoped to obtain some light on the matter by performing the above test, but inasmuch as the sensitised and unsensitised organisms react in the same way when they have been exposed to the action of aluminium and subsequently agglutinated in presence of NaCl and of NaCl in citrate, no information can be gained upon the point.

Had the sensitised organisms agglutinated in presence of citrate after they had been exposed to aluminium, one could have stated that the action of the aluminium alone differed greatly from the action of serum in presence of NaCl or other electrolyte with mono- or di-valent kation.

The chief difficulty arises from the fact that the organism suspension adsorbs both serum and aluminium and the dispersion of the latter by the citrate masks or is more potent than the agglutination of the sensitised bacilli by the salt.

Apart then from the meagre indication obtained from the experiments in which $TaCl_5$ was made use of, evidence is lacking that the phenomenon of sedimentation by the polyvalent kations is not due to the formation of hydroxides of the metals in a peculiar physical state, and therefore agglutination of unsensitised organisms with these reagents is probably not comparable to agglutination in presence of specific serum.

Beyond calling attention to this phenomenon and submitting the above suggestions, I wish to advance no further hypothesis concerning the action of these bodies in producing agglutination of sensitised and unsensitised organisms, as it is highly probable that even a slight alteration in the conditions of the experiment would lead to very different results, for we are dealing with a number of reagents whose affinities for one another, whose physical state, and whose " concentration " under any circumstances are all mutually interdependent.

The only conclusion then that can be definitely deduced from this section of the investigation is that the salts with polyvalent kations behave in respect of both sensitised and unsensitised organisms in a manner very different from the salts with mono- and di-valent kations. The mechanism of their

action is obscure and one experiences difficulty in correlating the result obtained with aluminium and other trivalent ions with that obtained with the pentavalent tantalum.

Experiments to investigate the Relation between the Concentrations of Serum and of Electrolyte.

Having determined that: (1) the precipitating value of an electrolyte acting on sensitised organisms is dependent on the valency of the kation of the electrolyte, and

(2) The titre of an agglutinating serum is no higher when a divalent kation is present in the precipitating fluid ($BaCl_2$ equimolecular with 0·9 % NaCl) than when a monovalent kation is employed (0·9 % NaCl) it seemed of interest to examine the relation of serum to electrolyte. It might well be that a low concentration of electrolyte would produce agglutination in presence of a given quantity of serum but that the reaction would not take place with the same concentration of the electrolyte in presence of less than that given quantity of the same serum.

To investigate this point, the following experiment was carried out.

Technique. An emulsion of organisms in distilled water was added to serum that had been diluted in NaCl and $BaCl_2$ to obtain the following concentration of the salts and dilutions of the serum when all the reagents were added to each tube.

TABLE XVIII. 2 *hours at* 37°.

NaCl					
N/20	N/40	N/100	N/200	N/400	Serum
+	+	+	+	+	1/100
+	+	+	+	+	1/500
+	+	+	+	?	1/1000
+	+	+	?	−	1/2000
+	+	?	−	−	1/4000
−	−	−	−	−	1/8000

$BaCl_2$					
N/400	N/500	N/666	N/1000	N/2000	
+	+	+	+	+	1/100
+	+	+	+	?	1/500
+	+	+	−	−	1/1000
+	+	−	−	−	1/2000
+	−	−	−	−	1/4000
−	−	−	−	−	1/8000

This shows that a relation of the nature indicated above does exist within certain limits; but the relation is probably not so simple as would appear from the above results, for $BaCl_2$ N/400 agglutinates in presence

of 1/4000 of the serum while in presence of $BaCl_2$ in concentration equal to N/6·5 (experiments of series 1) it only produces flocculation in 1/8000 of the same serum.

It is probable that as one approaches the dilution of serum which might be termed the " threshold value," the electrolyte has to be added in greatly increasing concentration in order to obtain aggregation of the bacteria.

Suggested Application of the above to the Differentiation of Bacterial Species.

It is possible that use might be made of the relation of serum to electrolyte for the purpose of differentiating closely allied bacterial species. Thus while the agglutinating " titre " of an anti-aertrycke serum might be the same for both *B. aertrycke* and for *B. paratyphosus b*, it seemed possible that if emulsions of these organisms were sensitised each in presence of 1/50 anti-aertrycke serum that the washed deposit in the case of the aertrycke bacilli sensitised with the anti-aertrycke serum might be flocculated by a lower concentration of the electrolyte employed than would the paratyphosus b. bacilli sensitised with similar concentration of the same serum. Or employing the serum as a constant throughout a series one might find that the anti-aertrycke serum in presence of aertrycke bacilli agglutinated with a lower concentration of electrolyte than did anti-aertrycke serum in presence of paratyphosus and *vice versa*.

The latter technique was adopted in the experiments. Employing anti-aertrycke serum in a dilution of 1/500 in presence of varying dilutions of NaCl to which were added emulsions of (1) typical *B. aertrycke*, (2) *Bacillus* " *D* "—a paratyphosus having peculiar reactions—and (3) typical *B. paratyphosus b*, the following result was obtained.

TABLE XIX. 1 *hour at* 37°.

Serum	NaCl	N/20	N/40	N/80	N/100	N/200	N/400
Anti-aertrycke	*B. aertrycke*	+	+	+	+	−	−
	Bacillus " *D* " ..	?	−	−	−	−	−
	B. paratyphosus b ..	?	−	−	−	−	−
	(titre of serum = 1/2000)						
Anti-paratyphosus b. ..	*B. aertrycke*	+	+	+	−	−	−
	Bacillus " *D* " ..	+	+	+	+	+	+
	B. paratyphosus b ..	+	+	+	+	+	+
	(titre of serum = 1/8000)						
Anti-" D "	*B. aertrycke*	+	+	+	−	−	−
	Bacillus " *D* " ..	+	+	+	+	+	+
	B. paratyphosus b ..	+	+	+	−	−	−
	(titre of serum = 1/4000)						

These results show that the specific antibody of the serum brings about agglutination in presence of a lower concentration of electrolyte than do the non-specific antibodies. Experiments with $BaCl_2$ corroborate the above results and I have applied the same technique in the examination of a number of strains of *B. aertrycke* and *B. paratyphosus b*, obtaining fairly constant results. The technique of the test, however, requires further elaboration before it could be satisfactorily applied to the differentiation of bacterial species.

Influence of Acid and Alkali on the Process of "Sensitisation."

The inhibiting action of alkalis and acids has been shown to be due to an interference with actual flocculation but it is possible that hydrion and hydroxidion also affect the process of sensitisation.

To investigate the action of acid in this connection a mixture of organisms suspended in saline, with HCl present in concentration equal to N/100, was sensitised with specific agglutinating serum 1/100. The mixture was allowed to stand at room temperature for 48 hours, it was then centrifuged, the supernatant fluid drawn off and the deposit washed with distilled water. This procedure was repeated twice and the material obtained was then suspended in distilled water.

This emulsion was added to a series of tubes containing the following reagents of such concentration that with the addition of the bacillary emulsion the dilutions shown in the following table were obtained.

TABLE XX. 2 *hours at* 37°.

	N/20	N/100	N/1000	N/10000	N/100000
NaCl ..	–	–	–	–	–
Na_2SO_4 ..	–	–	–	–	–
$Na_3Cit.$ ..	–	–	–	–	–
$BaCl_2$ ..	–	–	–	–	–

The above result appears to show that the presence of hydrion interferes with the union of antibody with antigen in addition to inhibiting the actual process of clumping.

The converse of the above experiment was carried out thus:

Technique. A saline suspension of organisms was rendered alkaline by the addition of N/10 NaOH till the concentration of NaOH was equal to N/100. To this was added agglutinating serum sufficient to make the serum

equal to 1/50 of the fluid. The mixture was allowed to stand for 48 hours, and was then centrifuged, decanted and distilled water added. It was afterwards shaken and the washing process repeated twice. The suspension was then exposed to the action of NaCl of the following concentrations.

TABLE XXI. 2 *hours at* 37°.

	N/20	N/50	N/100	N/200	N/400
NaCl ..	–	–	–	–	–

From these results it would appear that the reaction of the fluid has a most important influence on the process of sensitisation and that if it be too alkaline or too acid, the process is inhibited just as flocculation is prevented by the presence of hydrion and hydroxidion in certain concentrations.

I do not wish to lay too great stress on this series of experiments since, while the above appears to be true in the experimental conditions of the test, it is quite possible that a slight alteration of the conditions might have a profound effect upon the result obtained, and as I have so far not had the opportunity of repeating the experiments of this series, I hesitate to draw any definite conclusion from them.

Discussion of Results.

Reviewing the experiments that have been described, we see that, in the main, unsensitised organisms exhibit characters that are similar to those of fresh egg-white protein ; sensitised organisms on the other hand recall in their properties certain features characteristic of " denaturated " egg-white. Unsensitised organisms and fresh egg protein behave as do " non-rigid " colloids, while the reactions of sensitised organisms and " denaturated" egg-white in presence of electrolytes recall those of " rigid " colloids under the influence of these reagents.

I advance the hypothesis then that the action of antisera is probably akin to that of denaturation and this raises the question—" Why is it that in the case of certain bacterial species sensitised (by specific serum) the organisms are flocculated by electrolytes, while in the case of other species they are not so flocculated ? "

I cannot completely answer this question but if sensitisation be really akin to denaturation, we should almost expect that certain sensitised organisms would not be flocculated, for while denaturated egg-white is easily

flocculated by the addition of small quantities of electrolytes, serum protein on the contrary is very easily dispersed by the same reagents. The dispersion of egg-white is obtained only with difficulty by the use of salts and that only with polyvalent ions. I propose to investigate further this relation which I suggest exists between denaturation and sensitisation by performing a series of experiments similar to the above but employing anti-protein serum prepared by inoculating an animal with egg-white.

The fact that only certain serum-organism complexes show this peculiarity of flocculation is very strikingly shown in the case of the Bordet-Gengou bacillus of whooping cough [Bordet, 1909], in the case of which Bordet has, I think, shown conclusively that only certain antibody-antigen " complexes " are flocculable by electrolytes.

Looking at the question of bacteriolysis from the same standpoint, a number of important questions arise.

Thus, if the sensitisation of organisms to the agglutinating activity of electrolytes be a " denaturating " process, is the sensitisation of bacteria by specific serum to the dissolving activity of complement also a similar phenomenon ? In fact does a process similar to denaturation precede solution in the process of bacteriolysis or cytolysis ?

Arising out of the previous question a further problem presents itself—Is " complement " not a peculiar electro-physical state of some constituent or constituents of serum rather than a chemical " entity " ?

I am at present engaged on the investigation of this problem, but so far I have not sufficient evidence for or against the hypothesis to permit me to draw any conclusions from the work done, beyond stating that salts with divalent ions inhibit the dissolving action of complement upon red corpuscles while they certainly have no inhibitory effect upon the process of sensitisation of these by haemolytic serum.

In conclusion I should like to call attention to the important question of specificity of action of sera in connection with what one might call the colloidal theory of immunity. Specificity is well explained certainly by the side-chain hypothesis of Ehrlich, but it seems to me that it is capable of explanation otherwise.

I hesitate to advance any theory on this point, but we see from the above experiments that in one respect certainly—that of flocculation—immunity reactions (in vitro) are very delicate indeed and in view of the fact that an electrolyte becomes, in general, a more potent disturbing factor with increase in complexity of the molecule, may it not be that with bodies of very large

molecule, like bacterial protoplasm and serum, the adjustment of the physical conditions of the interacting substances is exceedingly delicate and that upon the delicacy of this adjustment depend the phenomena of immunity ?

Conclusions.

The following conclusions seem permissible from the experiments described.

(1) That in presence of salts with mono- and di-valent kations unsensitised organisms behave in a manner similar to " non-rigid " colloids, but that when sensitised they behave in respect of these reagents in a manner similar to " rigid colloids."

(2) That the factors determining specific agglutination are markedly altered by the presence of hydrion and hydroxidion in the suspending fluid.

(3) That the inhibitory action of these ions may be twofold, for they certainly inhibit flocculation and there is some evidence to show that they also interfere with the union of antigen with antibody.

(4) That—*caeteris paribus*—the character of the electrolyte has but little influence on the " titre " of the serum, provided always that it is sufficiently soluble and does not undergo hydrolysis.

(5) That when emulsions of bacteria that have been washed off directly in saline are used, the precipitating value of the electrolyte varies with the valency of the kation.

(6) That in acid suspensions, on the contrary, it varies with the valency of the anion.

(7) That the action of salts with polyvalent kations is peculiar in that these salts are as active in precipitating unsensitised organisms as they are in flocculating sensitised organisms.

No definite explanation of this is offered but it is suggested that the phenomenon of flocculation in these conditions depends upon a peculiar physical state of the metal hydroxides.

(8) That as unsensitised organisms behave in respect of the above points towards electrolytes as does fresh egg protein, but when sensitised have characters that recall those of denaturated egg-white, the process of sensitisation is akin to that of denaturation.

LITERATURE.

Beckhold (1904). *Zeitsch. physikal Chem.* **48**, 385.
Bordet (1899). *Ann. Inst. Past.* **13**, 225.
— (1909). Bordet and Gay, *Studies in Immunity*, p. 527. London, Chapman and Hall.
Burton (1906). *Phil. Mag.* (6), **12**, 472 and University of Toronto, *Studies from the physical laboratory*, No. 36.
Chick and Martin (1912). *J. Physiol.* **45**, 261.
Coplans (1913). *J. Pathology*, **17**, 367.
Gengou (1908). *Arch. inter. Physiologie*, **7**, 115.
Gruber and Durham (1896). *München. med. Wochensch.*, March.
Hardy (1899). *J. Physiol.* **24**, 172 and *Proc. Roy. Soc.* 1900, **66**, 110.
Krause (1897). *Wiener klin. Wochensch.*, No. 32.
Liefman (1913). *Centr. Bakt. Par.*, Ref. **75**, 14.
Linder and Picton (1895). *J. Chem. Soc.* **66**, **67**, 63.
Nicolle (1898). *Ann. Inst. Past.* **12**, 161.
Paal and Voss (1904). *Ber.* **37**, 3862.
Paltauf (1897). *Wiener klin. Wochensch.* No. 32.

XXXVIII. THE MODE OF OXIDATION OF CERTAIN FATTY ACIDS WITH BRANCHED CHAINS.

By HENRY STANLEY RAPER.

From the Department of Physiology, University of Leeds.

(*Received July 6th, 1914.*)

Our knowledge of the mechanism by which the simpler fatty acids with branched chains are oxidised in the body is still very meagre. This is no doubt due to the difficulty in interpreting the experimental results so far obtained, in any way that will lead to a generalisation such as we possess for the normal fatty acids; namely, β-oxidation followed by removal of the two end carbon atoms of the acid. This difficulty may be illustrated by reference to the behaviour of α-methylbutyric acid and α-ethylbutyric acid. In the diabetic organism the former gives rise to increased "acetone body" production [Baer and Blum, 1906] whereas the latter yields, in part at any rate, methyl propyl ketone [Blum and Koppel, 1911]. Similarly α-methylpropionic acid (isobutyric acid), gives rise to extra glucose formation [Ringer, Frankel and Jonas, 1913] and traces of *d*-lactic acid [Baer and Blum, 1906], a result interpreted by Baer and Blum as indicating that lactic acid is a probable intermediate stage in the oxidation of isobutyric acid, the methyl group being replaced by a hydroxyl group, thus:

$$CH_3 \cdot CH(CH_3) \cdot COOH \rightarrow CH_3 \cdot CH(OH) \cdot COOH.$$

This view gains support from the fact that lactic acid gives rise to extra glucose formation in diabetics. Replacement of the methyl group by hydroxyl is not general however, since α-hydroxybutyric acid, which might be expected by the above hypothesis as an intermediate product in the oxidation of α-methylbutyric acid, does not give rise to "acetone body" formation whereas α-methylbutyric acid does.

Another explanation of the behaviour of isobutyric acid has been advanced by Ringer [Ringer, Frankel and Jonas, 1913]. This observer showed that propionic acid, like isobutyric acid, produces increased glucose formation

in phlorizinised dogs. He suggests therefore that demethylation takes place by hydrolysis, methyl alcohol and propionic acid being formed. This is a type of reaction however that is at present unknown in organic chemistry and had therefore better be reserved as an explanation until proof is supplied that it can take place.

A third possibility has been suggested, namely, that the α-methyl group in such acids as isobutyric and α-methylbutyric, is oxidised to a carboxyl group. This view was abandoned by Baer and Blum, who first put it forward, when they discovered that ethylmalonic acid, which under this scheme would be produced from α-methylbutyric acid, does not cause increased "acetone body" formation in diabetics.

The object of the present communication is to suggest a mechanism of oxidation which applies to the α-substituted fatty acids and will explain the facts so far known about the catabolism of these acids. It is merely an application of the rule of β-oxidation advanced by Knoop and postulates that the carbon atom of the methyl group, which in these acids is in the β-position to the carboxyl group, is selectively oxidised, rather than the β-carbon atom of the main chain. Instead of a β-ketonic acid being produced therefore, as with the normal fatty acids, a derivative of the half aldehyde of malonic acid would result:

$$\begin{array}{l} \mathrm{CH_3} \\ | \\ \mathrm{R-CH\cdot COOH} \end{array} \rightarrow \begin{array}{l} \mathrm{CHO} \\ | \\ \mathrm{R-CH-COOH.} \end{array}$$

Malonic semi-aldehyde has been prepared by Wohl [1900] who showed that it rapidly lost carbon dioxide, especially on warming, producing acetaldehyde. In this way it behaves like the β-ketonic acids, which also easily lose carbon dioxide but give a ketone instead. No derivatives of malonic semi-aldehyde have been described but it is very probable by analogy with the derivatives of the β-ketonic acids, that they would be equally unstable. The second reaction therefore in the scheme suggested would be the loss of carbon dioxide with production of the *normal* aldehyde of an acid containing one carbon atom less than the original methylated acid. This aldehyde on further oxidation, or by the Cannizzaro reaction, could then give rise to the corresponding normal fatty acid:

$$\begin{array}{l} \mathrm{CHO} \\ | \\ \mathrm{R-CH-COOH} \end{array} \rightarrow \mathrm{R-CH_2-CHO} \rightarrow \mathrm{R-CH_2-COOH.}$$

In this way, isobutyric acid would give rise to propionic acid, α-methyl butyric to *n*-butyric acid and α-methylvaleric to *n*-valeric acid. In support of this view is the fact that the fate of these methylated acids in the body is exactly that of the corresponding normal acids which would be produced from them by demethylation. In the case of α-ethylbutyric acid there is no methyl group in the β-position for selective oxidation, and this acid appears to undergo β-oxidation in the usual fashion producing α-ethyl-acetoacetic acid and then methyl propyl ketone. The scheme of oxidation suggested thus provides an explanation of the fact that α-methylbutyric acid on oxidation in the body gives rise to acetone formation and not methyl ethyl ketone, which might be expected by analogy with α-ethyl butyric acid [Blum and Koppel, 1911].

The hypothesis put forward above, to explain the mode of oxidation of the α-methylated acids, will also apply to those acids, such as isobutyl-acetic, which contain a methyl group in the γ-position, since by undergoing the usual β-oxidation they yield α-methylated acids. The mode of oxidation of β-methylated acids, such as isopropylacetic acid, cannot yet be explained by a simple scheme such as that suggested for the α-methylated acids. The reactions involved in their catabolism must be left for further experiment.

It has been shown by Dakin [1908] that on warming the ammonium salts of the normal fatty acids with dilute hydrogen peroxide, β-oxidation is regularly observed in all the cases investigated from butyric to stearic acids. A ketone can be isolated from the products of oxidation which is derived from a β-ketonic acid. Since in hydrogen peroxide we have an oxidising agent known to be capable of producing β-oxidation it has been employed in the first instance in this investigation to determine whether the type of oxidation represented above can actually take place with iso-butyric acid and α-methylbutyric acid. If the scheme suggested for the oxidation of these acids be correct, then isobutyric acid should give propion-aldehyde as an oxidation product and similarly α-methylbutyric acid should give butyraldehyde. Evidence has been obtained that the ammonium salts of these acids on oxidation with hydrogen peroxide do yield propionaldehyde and butyraldehyde respectively. Direct identification of the aldehydes was not possible, because of the simultaneous production of ketones resulting from oxidation at the α-carbon atom, acetone being obtained in the first case and methyl ethyl ketone in the second. The aldehydes were therefore oxidised to the corresponding acids and these isolated as silver salts. In addition to butyraldehyde, acetaldehyde was regularly observed as an

oxidation product of α-methylbutyric acid. The mechanism of its production appears to be somewhat obscure.

It is worthy of note that propionic acid is similarly constituted to the α-methylated fatty acids in that its β-carbon atom forms part of a methyl group, and it might be expected therefore to undergo the same kind of oxidation as the α-methylated acids. In that case it would yield malonic semi-aldehyde:

$$CH_3 - CH_2 - COOH \rightarrow CHO - CH_2 - COOH.$$

We already possess a certain amount of evidence in support of this view. It is certain for instance that β-phenylpropionic acid undergoes β-oxidation yielding benzoylacetic acid, and not α-oxidation which would yield phenyl-pyruvic or phenyl-lactic acid. Further, on oxidation with hydrogen peroxide, propionic acid yields a large amount of acetaldehyde [Dakin, 1908], although this might equally well be explained by α-oxidation, with the intermediate production of lactic acid, and not malonic semi-aldehyde. Ringer has observed that in phlorizinised dogs, propionic acid gives rise to extra glucose formation. No experiments have been performed with malonic semi-aldehyde but quite recently Ringer and Frankel [1914] have stated that acetaldehyde produces extra glucose formation in diabetic dogs. Since the administration of acetaldehyde causes a marked depression in the nitrogen excretion and the calculation of the extra glucose formed depends on the G/N ratio, the interpretation of these authors cannot be accepted unreservedly. It is noteworthy however that in spite of this variation an absolute rise in the glucose excretion was observed.

Experimental.

Oxidation of Ammonium Isobutyrate. Kahlbaum's isobutyric acid was used. Portions of ten grams were neutralised with ammonia, the solution diluted with about 100 cc. of water and twenty grams " perhydrol " added, the volume being then finally brought to about 200 cc. by further dilution with water. The oxidation was carried out by warming this solution in a flask connected with a well cooled condenser, the receiver being immersed in ice and salt. During the experiment a gentle current of air was aspirated through the apparatus in order to assist in the removal of the volatile products of oxidation from the flask in which the oxidation was carried out. The temperature was raised fairly rapidly to 70° and thereafter more slowly to boiling point. The heating was continued for half to one hour at this point

and the flame was maintained at such a height that distillation was very slow. The distillate, which was always alkaline, owing to the ammonia present, was redistilled to free the volatile oxidation products from possible traces of isobutyric acid. Direct isolation of a pure *p*-nitrophenylhydrazine derivative from the distillate was impossible, no substance with a sharp melting point being obtainable. The presence of aldehydes was easily detected by Tollen's reagent which was immediately reduced in the cold. Dakin [1908] using a slightly different method has shown that acetone is produced when ammonium isobutyrate is distilled with hydrogen peroxide. In order therefore to identify the aldehyde or aldehydes present, the distillate was oxidised with ammoniacal silver oxide containing a trace of sodium hydroxide. Oxidation was carried out at the ordinary temperature for several hours and then overnight in the incubator at 37°. The precipitated silver was filtered off, the filtrate nearly neutralised with phosphoric acid and distilled to remove the ketone. The distillate still contained a little aldehyde. This was removed by adding a few cc. of permanganate solution and redistilling. On treating the distillate with *p*-nitrophenylhydrazine acetate an immediate precipitate of hydrazone was obtained, which melted sharply at 148–9° after two recrystallisations from alcohol. This is the melting point of acetone-*p*-nitrophenylhydrazone. Dakin's observation of the formation of acetone was thus confirmed. The alkaline liquid from the oxidation with silver oxide, after removal of the acetone by distillation, was acidified with phosphoric acid and the volatile acids, corresponding to the aldehydes in the original distillate, distilled off. The distillate was neutralised with baryta and evaporated to dryness. The barium salts were washed with a little watery alcohol to remove barium acetate if present, then dissolved in a small amount of water and the solution left to crystallise. The crystalline barium salt was converted into the silver salt by precipitation with silver nitrate, washed with a little distilled water and dried in vacuo over sulphuric acid. The silver salt on analysis proved to be silver propionate.

0·1938 g.; 0·1165g. Ag = 60·1 %.
Calculated for $C_3H_5O_2Ag$ 59·7 %.

Propionic aldehyde was therefore one of the oxidation products of isobutyric acid.

Non-volatile Oxidation Products. The residual liquid from the peroxide oxidation contained much unchanged isobutyric acid. It was tested specially for formic acid by the mercuric chloride reaction and this acid was

present in traces. After removal of the isobutyric acid by continuous extraction with petroleum ether, the liquid was extracted with ether but no methylmalonic acid could be isolated from this extract.

Oxidation of Ammonium α-Methylbutyrate.

The acid used was prepared from methylethylmalonic acid and partly from methylethylacetoacetic ester. The oxidation was carried out in the same way as that of the isobutyrate. The distillate containing the volatile oxidation products did not yield a pure *p*-nitrophenylhydrazine derivative so that a separation of the aldehydes and ketone by means of ammoniacal silver oxide was undertaken. In this way a ketone was obtained which gave a *p*-nitrophenylhydrazone crystallising in orange-coloured needles and melting sharply at 124–124·5°. This was found to be the melting point of the *p*-nitrophenylhydrazone of methyl ethyl ketone prepared from methylacetoacetic ester[1]. A mixture of the two derivatives showed no change in the melting point. The acids obtained from the aldehydes by oxidation with silver oxide were isolated as barium salts which did not crystallise but were easily soluble in 95 % alcohol. The acids were therefore liberated by means of phosphoric acid, distilled off and converted into the calcium salts by neutralising the distillate with lime water. These crystallised readily on evaporating the solution and had the characteristic flocculent appearance of calcium acetate. The calcium salt was transformed into the silver salt and analysed.

0·1286 g.; 0·0781 g. Ag = 60·7 %.
∴ Mean molecular weight of acids = 70.

The mean molecular weight of the acids lay therefore between that of propionic acid (74) and acetic acid (60). Propionic acid was not present in any considerable amount however as the barium salts of the mixed acids were easily soluble in 95 % alcohol, whereas barium propionate is almost insoluble. The acetic acid undoubtedly present was therefore mixed with a higher acid. That butyric acid was present was easily demonstrated by warming a small portion of the calcium salts with sulphuric acid and methyl alcohol when the characteristic odour of methyl butyrate was obtained. It was thought desirable to obtain more exact evidence of the presence of butyric acid. A trial with equal amounts of calcium acetate and butyrate

[1] Dakin [1908] gives 128—9° as the melting point of the *p*-nitrophenylhydrazone of methyl ethyl ketone, prepared by him, but does not state the source of the ketone.

dissolved in water and evaporated, showed that separation could not be obtained satisfactorily with 95 % alcohol, in which calcium acetate is practically insoluble and calcium butyrate easily soluble, due no doubt to the formation of a mixed salt with intermediate solubilities. Distillation of the free acids was therefore used as a means of separation. The acids obtained from several experiments were obtained as sodium salts, and the free acids then liberated by sulphuric acid and taken up in a small amount of petroleum ether. The petroleum ether and acetic acid were then removed by distillation up to a temperature of 155°. The residual acid was dissolved in water and converted into the calcium salt by boiling with calcium carbonate. The calcium salt, which was easily soluble in 95 % alcohol, was converted into the silver salt which was dried in vacuo over sulphuric acid and on analysis proved to be silver butyrate probably still containing traces of the acetate.

0·2245 g.; 0·1255 g. Ag = 55·9 %.
Calculated for $C_4H_7O_2Ag$ 55·4 %.

Non-volatile Oxidation Products. As in the case of isobutyric acid traces of formic acid were detected by the mercuric chloride reaction. After removal of unchanged α-methylbutyric acid by distillation in steam, the residual liquid was neutralised, concentrated on the water bath, then acidified with phosphoric acid and extracted with ether for twelve hours. The ether extract contained a crystalline acid which, on recrystallisation from benzene containing a little acetone, and then from water, melted at 115°. This proved to be methylsuccinic acid. On heating with ammonia and zinc dust the pyrrole reaction was easily obtained.

0·1196 g. required 17·89 cc. 0·1 N. NaOH for neutralisation. Equivalent = 66·8.
Calculated for $C_3H_6(COOH)_2$ 66.

No evidence of the presence of ethylmalonic acid was obtained.

Summary.

1. A scheme of oxidation is suggested which explains the results so far obtained in studying the catabolism of α-methylated fatty acids such as isobutyric, α-methylbutyric and α-methylvaleric acid, and also acids such as γ-methylvaleric acid which by β-oxidation would yield α-methylated acids.

2. The rule of β-oxidation is applied to these acids with the proviso that the carbon atom of that methyl group which is in the β-position undergoes oxidation first. The derivative of malonic semi-aldehyde produced,

would, by loss of carbon dioxide give rise to the *normal* aldehyde of the demethylated fatty acid and by subsequent oxidation, or the Cannizzaro reaction, to the normal acid itself.

3. In support of this view as a possible biochemical process it is shown that ammonium isobutyrate on oxidation with hydrogen peroxide yields amongst other products propionaldehyde, and that ammonium α-methylbutyrate yields similarly butyraldehyde.

4. The possibility of this scheme being applied to the catabolism of propionic acid is suggested, since this acid is similarly constituted as regards the position of its methyl group to the α-methylated acids.

REFERENCES.

Baer and Blum (1906). *Arch. exp. Path. Pharm.*, **55**, 89.
Blum and Koppel (1911). *Ber.*, **44**, 3576.
Dakin (1908). *J. Biol. Chem.*, **4**, 227, 236.
Ringer and Frankel (1914). *J. Biol. Chem.*, **16**, 563.
Ringer, Frankel and Jonas (1913). *J. Biol. Chem.*, **14**, 525.
Wohl (1900). *Ber.*, **33**, 2763.

XXXIX. THE CHEMICAL NATURE OF A BACTERIAL HAEMOLYSIN.

By ERIC EDWIN ATKIN.

From the Bacteriological Department, Lister Institute.

(*Received July 7th, 1914.*)

I. The relation between the velocity of Heat-destruction of a Bacterial Haemolysin and the True Reaction of the Substrate.

Our present knowledge of the effect of heat, and also of acid and alkali on the velocity of destruction of bacterial haemolysins, is due to Famulener and Madsen [1908]. They were able to show that in all cases, whether by altering the temperature or the degree of acidity or alkalinity of the medium, the process of destruction proceeded as a reaction of the first order—that is to say, the destruction at any instant was proportional to the amount of haemolysin then present. They found that as they increased the quantity of acid or alkali, the velocity constant increased and there was a minimum velocity somewhere in the neighbourhood of the neutral point. The exact relationship of this minimum to neutral reaction of the medium, they did not determine however, as the hydrogen ion concentration was not measured. Since that time the technique of the determination of the hydrogen ion concentration of biological liquids by the electrical method has greatly developed and at the present day we can find its value with a very considerable degree of accuracy. It is therefore interesting to investigate how the velocity of destruction varies with the hydrogen and hydroxyl ion concentration. The fact that the velocity increases both with acidity and alkalinity is strongly in favour of the view that the substance producing haemolysis is an amphoteric electrolyte and that both anion and cation are thermolabile, while the undissociated molecules are probably not at all so, under the conditions of the experiment. On this assumption the velocity should be at a minimum when the anions and cations are present in the fewest possible numbers, that is at the isoelectric point. From such experiments therefore

we ought to obtain information about the relative magnitudes of the acidic and basic dissociation constants of the haemolysin. We shall then know whether the haemolysin functions as a weak acid or base in pure watery solution.

The haemolysin I have used is that produced in broth cultures of *Vibrio nasik*. After growing this micro-organism in alkaline broth (such as is employed for the production of diphtheria toxin) for a period of nine or ten days at 37°, it is killed by toluene and the dead bacteria removed by a centrifuge at a speed of about 5000 revolutions per minute. The clear liquid is separated and toluene added. The haemolysin keeps well at room temperature. It is lytic for the red corpuscles of many different animals. I have used a 1 % suspension of sheep red cells in 0·9 % NaCl. The relative amounts of haemolysin present in a series of samples were estimated by Madsen's colorimetric method [Kraus and Levaditi, 1908].

The reaction of nasik lysin was altered by the addition of HCl or NaOH according as an acid or alkaline medium was required. Small amounts of acids simply had the effect of neutralising or lessening its degree of alkalinity —the lysin being alkaline to begin with. In each case a definite amount of acid or alkali was added to 90 cc. of lysin and the whole made up to 100 cc. with distilled water. It was then left overnight in the cold room and next day hydrogen was bubbled through for three hours. This procedure dissipated the traces of toluene which were still dissolved in the lysin, and which Sörensen has shown may adversely affect the electrodes in the electrical method of estimating hydrogen ion concentration. It also had the effect of removing any excess of CO_2 or NH_3. Carbon dioxide is not an electrically active gas, that is to say, it does not give rise to an electromotive force in a platinum electrode dipping in a liquid, but when dissolved in water it forms H_2CO_3 which is slightly dissociated into $H^{\cdot}$ and HCO_3' ions and therefore increases the acidity of the solution. This would not matter except for the fact that the CO_2 easily diffuses out of the solution, notably into the atmosphere of hydrogen during electrical measurement of the reaction, thereby altering the reaction of the liquid and the saturation of the electrode. Ammonia on the other hand is electrically active and would modify the potential of the hydrogen electrode. In the most alkaline samples of lysin with which I worked there was a very distinct smell of NH_3, due no doubt to decomposition of ammonium salts by the added NaOH and so it was very necessary to get rid of this. The lysin was therefore left in contact with hydrogen for another day in the cold room. The most suitable temperature at which the destruction of the lysin could be followed, was shown by

Famulener and Madsen's experiments to be about 46·0° and this temperature was therefore chosen. Immediately before the heating of the lysin took place, its hydrogen ion concentration [H˙][1] was measured by the electrical method. The particular type of apparatus used was that described by Michaelis and Rona [1909] the characteristics of which are the *still* atmosphere of hydrogen combined with *minimum immersion* of the electrode. When the electrodes only just dip into the liquid the E.M.F. becomes constant very quickly and this is often of great importance in biological work where the reaction may be altering during the operation. The measurements took place at 46·0°—the same temperature as that at which the destruction of lysin was followed. As normal electrode I used a hydrogen electrode dipping into N/10 HCl. The [H˙] of this solution at 46° is only slightly less than at ordinary room temperature and it was estimated by graphic interpolation from data given by A. A. Noyes and others [1910] to be dissociated to the extent of 91·4 %. As connecting liquid KCl was used and readings taken with two strengths, namely, 1·75 N and 3·5 N solutions and the true value extrapolated by addition of the difference of the two potentials to the latter according to the method suggested by Bjerrum [1905]. About two minutes was allowed for the cells to attain the temperature of the water bath and then readings taken immediately. Potential equilibrium is practically established by this time as one would expect, especially as the hydrogen electrodes have been in contact with the liquids to be tested for some minutes previous to immersion in the water bath. By continuous observation of the E.M.F. at 46° a very gradual change is perceived due to slight alterations of the reaction of the lysin, as will be mentioned later. An immediate reading as soon as the lysin has reached 46° is necessary therefore and although an error may creep in here it can only be trifling.

The reaction of a given sample of lysin being ascertained, it was next heated to 46·0°. For this purpose a water bath with a toluene regulator was employed. During the heating the temperature never varied more than ± 0·025° and often was practically constant, the thermometer used being an accurate one graduated in tenths of a degree and read with the naked eye. Arrhenius has introduced an empirical formula for the relation between velocity constants and temperature, namely,

$$\frac{k_1}{k_2} = e^{\frac{\mu}{2}\left(\frac{T_1 - T_2}{T_1 T_2}\right)},$$

[1] H˙ stands for hydrogen ion, the brackets for concentration, and p_H for the negative exponent when the normality is expressed as a power of 10.

k_1 and k_2 being the velocity constants at the absolute temperatures T_1 and T_2 and μ a constant for a given substance. Famulener and Madsen [1908] have shown that this equation is valid for nasik haemolysin, where $\mu = 128{,}000$. We can therefore calculate what would be the change in the velocity constant for a rise in temperature from 45·975° to 46·025°. Under these conditions the ratio $\frac{k_1}{k_2} = 1{\cdot}032$, which represents the maximum error and is so small as to be negligible. The lysin was placed in a large test tube containing the thermometer, which was also used for stirring, and fitted with a large cotton wool plug. It was previously heated to about the required temperature and quickly transferred to the water bath. As soon as the thermometer registered exactly 46·0° a sample was removed by insinuating a narrow bore glass tube at the side of the wool plug, the top of which was fitted to a 10 cc. pipette with a piece of rubber tube. From the pipette 7 cc. were measured off into a tube containing sufficient acid or alkali to neutralise the added alkali or acid, and immediately cooled down in ice-cold water. By this manœuvre the temperature of the lysin in the water bath did not appreciably alter. The attempt to bring the reaction back to its original value by adding the calculated amount of acid or alkali as the case might be, was only partially successful as can be easily imagined, but this is not of great importance since the haemolytic action of the lysin on red cells, within fairly wide limits, is independent of the [H·]. After seven samples had been removed at definite intervals of time the [H·] of the remainder was determined as before. It was found that in practically every case the reaction had changed during heating to a greater or less extent. It might be thought that it would have been better to alter the reaction by means of regulator mixtures which keep the reaction constant, that is, weak acids or bases in conjunction with their salts, instead of HCl and NaOH. There are however objections to this. In the first place I was desirous of introducing, if possible, only ions of the kind already present. Evidence is accumulating that other anions and cations besides H ions and OH ions, influence the chemical actions of biological substances and so may have altered the chemical relation of the lysin to H and OH ions. In addition, the amount of regulator necessary would be relatively large, and the dilution of the lysin would be such as materially to limit the range over which the destruction could be studied. As can be seen from Fig. 1 the alteration of reaction during heating is in most instances relatively slight and in any case insufficient to vitiate the main results of this investigation. Fortunately the greatest changes took place

in the neighbourhood of the neutral point, where considerable alterations in the reaction produce relatively small changes in the velocity constant.

The following will serve as a type of a single determination of a velocity constant :

1 N.HCl	H_2O	Nasik lysin	[H·]
2·2 cc.	7·8 cc.	90 cc.	p_H. at 46·6°
			5·47

Heated at 46·0°.

Minutes	Haemolytic Equivalents. Volumes of lysin producing equal haemolysis	$\left(k=\frac{1}{t_2-t_1}\log_e\frac{A-x_1}{A-x_2}\right)$ $k\times 0{\cdot}4343$
0	0·055	
15	0·065	0·00484
30	0·07	0·00214
45	0·072	0·00082
60	0·075	0·00118
75	0·09	0·00528
90	0·11	0·00581
	Mean	0·00335

To 7 cc. of each sample after heating were added 0·154 cc. of N.NaOH and 0·846 cc. of water. After heating at 46°, p_H. was found to be 5·51.

TABLE I. *Velocity of Destruction of Nasik Lysin at Different Reactions.*

$\left(k=\frac{1}{t_2-t_1}\log_e\frac{A-x_1}{A-x_2}\right)$ $k\times 43{\cdot}43$	p_H. at 46·0°	
	before heating	after heating
28·0	8·43	8·49
18·93	8·30	8·23
15·7	8·01	8·17
9·84	8·19	8·10
5·2	7·55	7·62
2·95	7·37	7·17
0·72	7·06	6·95
0·4	7·06	6·62
0·08	6·54	—
0·22	6·46	6·18
0·16	5·82	5·49
0·335	5·48	5·5
2·05	5·17	5·07
3·95	5·04	5·04
6·4	5·10	4·88
10·6	5·00	4·82
16·95	4·77	—

In this way a number of values for k were obtained between two limits of [H·] in each case. The results have been tabulated and charted. An inspection of Fig. 1 will reveal the points of interest.

The lines through which the curve has been drawn represent the limits of the change of reaction during heating. The velocity of destruction rises

with increasing acidity and also with increasing alkalinity though not so abruptly in the latter case. The minimum does not correspond with the neutral point but is slightly on the acid side.

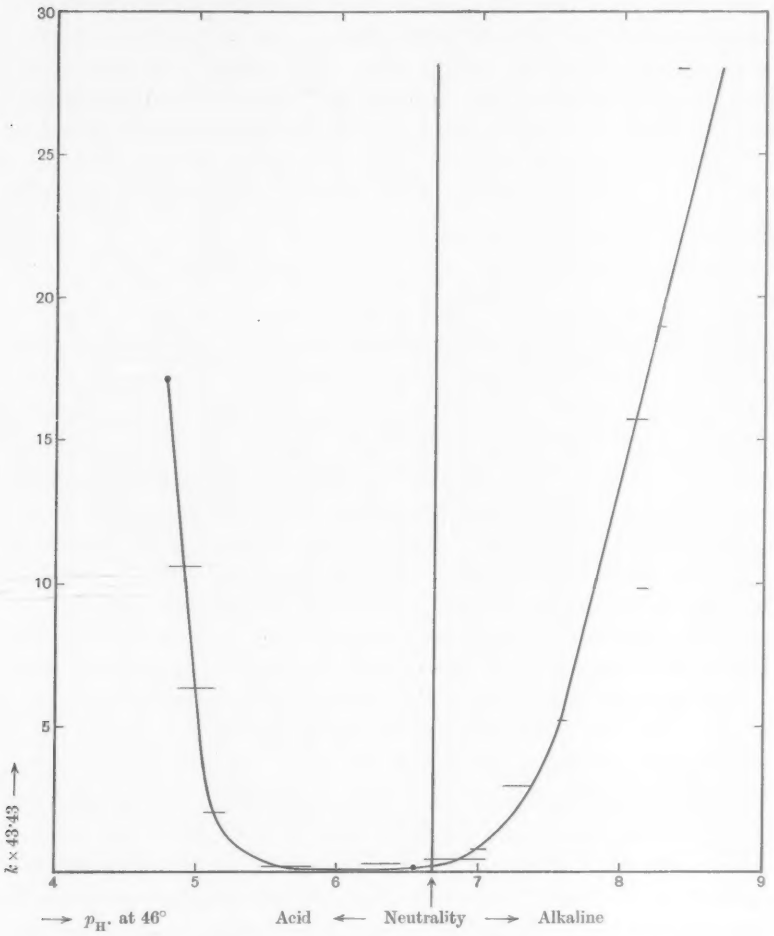
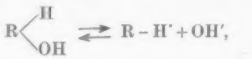


Fig. 1. Relation between Velocity of Destruction of Nasik Haemolysin and the Reaction of the Substrate at 46·0°.

Interpretation of Results.

These facts can best be explained on the assumption that the substance causing haemolysis is an amphoteric electrolyte with an acid dissociation constant greater than the basic, that is $k_a > k_b$. If we represent an ampholyte (amphoteric electrolyte) in the usual way

then in strongly acid solution it will be ionised practically entirely thus:



and in strongly alkaline solution

Both the anion and cation are thermolabile and we should expect that the velocity of destruction would be at a minimum at the isoelectric point (I.P.) because here the total number of anions and cations is a minimum. The I.P. is by definition the $[H^{\cdot}]$ of the medium when the anions and cations of the ampholyte are present in equal numbers and it can also be shown that their total concentration is the least possible at a given temperature and in a given solvent—in other words at the I.P. the molecules of an ampholyte suffer least dissociation in solution. It is easy to see that if the $H^{\cdot}$ and OH' have the same tendency to split off from the molecule ($k_a - k_b$) the I.P. will correspond exactly with the neutral point; but if, for example, there is a greater tendency for the $H^{\cdot}$ than the OH' to dissociate, as is the case with so many protein substances, it will be necessary, in accordance with the law of mass action, to add an acid to limit the splitting off of H ions in order that an equal number of anions and cations may be present. Now the neutral point at 46·0° obtained by graphic interpolation from data for the dissociation product of water at different temperatures given by Lundén [1908] is $p_{H^{\cdot}}$ 6·69. On reference to Fig. 1 the minimum of the curve appears well on the acid side of the neutral point, and the explanation is that the lysin has a larger acid than basic dissociation constant. These results are consistent with the view that the lysin is an ampholyte comparable with the serum proteins.

There is another possibility, however, namely that the lysin is a dibasic acid. A minimum velocity of destruction would then correspond with a

maximum number of monovalent anions present, and the velocity would increase on the one hand with the number of undissociated molecules of lysin, and on the other hand with the divalent anions, according as the solution was made more acid or less acid respectively.

Trypsin is an ampholyte in which only the monovalent anion has a ferment action, but it seems probable that divalent anions which are inactive are formed by splitting off of another $H^{\cdot}$ on further reducing the acidity [Michaelis and Davidsohn, 1911]. Similar considerations would apply in the case of a di-acid base. It is only possible to choose between the two alternatives by testing the direction of movement in an electric field, in solutions of varying $[H^{\cdot}]$.

II. Cataphoresis experiments with Nasik Lysin.

The lysin already employed was not suitable for this purpose on account of its high electrical conductivity. The original broth on which the vibrio was cultivated contained 0·5 % NaCl in addition to salts from the meat and peptone. I first attempted to produce a lysin in a salt-poor medium—peptone water alone or with a little broth added—but I only succeeded in obtaining a weak lysin in this way. Dialysis was next tried. Parchment paper proved useless as the lysin disappeared almost as quickly as the salts from the solution. Much better results followed the use of an animal membrane as dialyser (the so-called Fischblase), which has been successfully used to obtain electrolyte-free ferment solutions. By dialysing for 24 hours against distilled water, I was able to get a fairly strong lysin with an electrical conductivity about quarter that of N/100 NaCl. Reduction of lytic power occurred here also, but to a much less extent than in the case of the parchment membrane.

The apparatus used was that designed by Landsteiner and Pauli, with unpolarisable electrodes of Ag in NaCl, and Cu in $CuCl_2$ which have been recommended by Michaelis. In order to vary the $[H^{\cdot}]$ a regulator mixture was used as it did not seem probable that the reaction would remain sufficiently constant during the experiment by the simple addition of HCl. It was not necessary to make the solution more alkaline than it already was, as the lysin migrated to the positive pole without any addition.

The most suitable mixture to produce the required range of $[H^{\cdot}]$ was lactic acid and its sodium salt. This regulator has been used several times in the case of ferments and the lactate ion has not been found to have

any specific effect. This regulator was added to the lysin in the ratio of regulator : lysin = 1 : 9. It is not practicable to dilute the lysin more as its strength would be insufficient to make it easy to detect when it had moved to one or other pole in small quantity. Its content of added salt (sodium lactate) was 0·02 N. This is not very concentrated for a regulator but it seemed desirable to keep the electrolyte content as low as possible. The slaking power of the lysin on the [H·] of the regulator was considerable. After several mixtures had been made and the values of their [H·] estimated it was easy to construct a curve from which to predict fairly accurately in advance the [H·] of any mixture. Such mixtures were put in the middle compartment of the cataphoresis apparatus, and in the side pieces a regulator mixture of as nearly as possible the same [H·], with the lysin replaced by distilled water. The latter mixture was therefore of slightly lower specific gravity than the lysin mixture, but had the same amount of added electrolyte, if we neglect the slightly dissociated anions of the lactic acid. The electrodes were filled with 0·01 N NaCl in addition to the small amount of concentrated salt solutions—NaCl on the positive side, and $CuCl_2$ on the negative side—which formed layers at the bottom. The whole apparatus when filled was air-tight, and was sunk in a water bath at 10°. A pressure of 200 volts was then applied for four hours. The current passing was of the order of 0·25 milliampere, but varied of course with different conditions of the solutions, the concentration of electrolyte at the electrodes and the size of the electrodes. At the end of the time allowed, 0·4 cc. of liquid was removed from the bottom of each side tube of the apparatus with a Jena glass pipette, which was drawn out to a very fine capillary and was fitted to a syringe by means of a small rubber cork. Very evenly regulated slow suction was produced in this way, so that the bottom layer of liquid just above the taps in the side tubes could be abstracted without mixing with the supernatant layers. To this liquid was added 0·2 cc. of a 3·6 % solution of NaCl, to bring its tonicity up to the level which the red blood cells can tolerate without becoming haemolysed. It was then carefully neutralised with 0·2 N NaOH. The technique of neutralisation presented some difficulties at first owing to the fact that it was not feasible to waste more than a minute amount of liquid in the process, and it was very necessary to bring the solution near neutrality or else haemolysis would be produced by the excess of acid or alkali present. Very small drops were taken and tested with equally small drops of suitable indicators such as methyl-red, rosolic acid, α-naphthol-phthalein, on a white porcelain plate. It was found

absolutely essential to use Jena glass for the pipettes for removal of the liquid and indicators. In this way it was easy to neutralise with a very small total loss of liquid. Lastly 1·0 cc. of a 1 % suspension of sheep red cells in 0·9 % NaCl was added, and the whole incubated for three hours at 37°. The tubes were then shaken and examined next day for haemolysis. A similar sample was always taken from the middle compartment at the end of cataphoresis to show that the lysin had not been destroyed by excessive acidity or other circumstances (see later). In all the mixtures of lysin and regulator a turbidity appeared immediately which gave rise to more or less precipitate as time went on, according to the degree of acidity.

This precipitate is presumably mainly composed of denaturated protein coming from the meat used in the preparation of the original broth, which only completely flocculates when brought to its isoelectric point. At any rate a similar precipitate can be obtained from a meat broth (without peptone) with its optimum at about the same [H$^{\cdot}$]. The presence of this protein impurity in the lysin solution was a considerable complication. At certain degrees of acidity it had the power of removing much of the lysin from the solution, in the process of precipitation, leaving only a weak lysin to work with. Before discussing its effect on the direction of migration of the lysin I will indicate the experimental results.

Table II shows the direction of movement of the lysin in an electric field at varying [H$^{\cdot}$]. The temperature was 10° and the [H$^{\cdot}$] was determined at the same temperature using in this case a calomel electrode as standard. The π_0 value (0·3383) at 10^5 was obtained by graphic extrapolation from values given by Sörensen [1912]. It will be seen that in most cases there is not very much difference between the [H$^{\cdot}$] of the lysin before and after cataphoresis. The liquid on the + side in every case became more alkaline ; that on the − side sometimes increased in acidity but showed less tendency to change from the original reaction of the regulator. It is obvious that these small deviations from the reaction of the lysin in the middle compartment will not militate against the definition of the limit of movement to either pole. For instance a lysin anion travelling towards the + pole on coming in contact with a more alkaline medium will not tend to have its charge reversed as it would if it met a more acid medium, and it will proceed on its way. Similarly on the acid side increased acidity will not interfere with the progress of the lysin cations. The charge and therefore the direction of movement of the ions will thus be determined by the [H$^{\cdot}$] of the lysin. It will be seen that movement to both poles has been obtained

TABLE II. *Cataphoresis of Nasik lysin*

Temperature 10°
Duration, 4 hours

E.M.F. 200 volts.

k_w at 10°, $10^{-14.53}$

No.	Lysin, cc.	0·1 N. Sod. lactate, cc.	0·1 N. Lactic acid, cc.	H_2O cc.	p_H Before cataphoresis: Lysin	Regulator	p_H After cataphoresis: Lysin (middle compt.)	+side	−side	Movement Pole	Remarks
1	9	0·5	0·075	0·425	3·92	4·00	3·87	4·23	3·88	+	
2	,,	,,	0·1	0·4	3·77	3·94	3·85	4·28	3·92	+	
3	,,	,,	0·15	0·35	3·60	3·60	3·59	3·85	3·63	+	+liquid turbid.
4	,,	,,	0·2	0·3	3·29	3·26	3·23	3·55	3·25	0	No movement of visible particles. Strength of lysin after exp. much reduced.
5	,,	,,	0·4	0·1	3·01	2·87	3·01	3·02	2·9	0	
6	,,	,,	0·5	0	2·94	2·99	2·92	3·10	2·97	−	−liquid turbid.
7	,,	,,	0·5	0	2·94	2·70	—	2·9	2·78	−	
8	,,	,,	0·7	0	2·75	2·73	2·82	3·02	2·74	?	All lysin destroyed at end of exp., due to extreme acidity.

with a zone of no movement between them, according to the degree of acidity. This points to the fact that the lysin is an ampholyte and lends additional support to the interpretation of the experiments on velocity of destruction of the lysin by heat.

Discussion.

The early experiments to determine the electric charge of antigens (toxins, etc.) were all vitiated by neglect to exclude sharp changes of reaction at the electrodes. Field and Teague [1907] used platinum electrodes and in addition complicated their work by the presence of agar, through which they designed the toxin should travel and lend itself to easy detection. They determined that both the toxin and antitoxin of diphtheria and tetanus had positive charges which were not reversed by altering the reaction of the solvent—a most unlikely result in the light of later research. Teague and Buxton [1907] believed that they had shown that haemagglutinins moved to the cathode and considered that "all the active principles of serum concerned in the anti-reactions have a tendency to migrate towards the cathode." Their experiments were carried out in a similar way to those of Field and Teague except that they used unpolarisable electrodes. Bechhold [1907] used membranes with the idea of excluding the products formed at the poles from the substance being examined.

Landsteiner and Pauli [1908] in the case of abrin, ricin, and the haemagglutinin found in normal fowl serum, obtained a movement to the negative pole in each. A determination of the $[H^{\cdot}]$ of the solution was here lacking, but it was presumably not far from the neutral point. These experiments are an advance on the previous ones as the fallacies of the method were eliminated.

Landsteiner [1913] states that he has some unpublished results showing that staphylolysin and arachnolysin are amphoteric. The only details given are that in weak acid reaction the lysins migrated to the negative pole, and in weak alkaline to the positive pole.

Let us now attempt to co-ordinate the two series of results, namely, the curve of velocity of destruction and the movement in an electric field. The temperature in the two cases was different because the minimum velocity of destruction of the lysin had to be reduced for the cataphoresis experiments, so that the lysin should not be appreciably weakened by the end of the time allowed. Suppose for the present we estimate the I.P. at 10° (Table II) at about p_H 3·3. At 46° the minimum velocity of destruction

can be read from the curve at roughly p_H· 6·1. Is it reasonable to suppose such a difference in the I.P. would occur at the two temperatures? In the first place it must not be forgotten that values of [H˙] for different temperatures are not directly comparable. For instance at 46° the [H˙] of neutral reaction is p_H· 6·69, but at 10° the [H˙] of neutral reaction is p_H· 7·26. It is obvious that these two values are thermodynamically equivalent. It is in fact the ratios H˙/OH′, which correspond at two different temperatures, this ratio being of course equal to unity at neutrality. A simple calculation shows that the ratios of H˙ to OH′ will be the same at the two temperatures when the p_H· is 6·1 at 46° and 6·67 at 10°. In other words there is a difference of I.P. corresponding to the difference between p_H· 6·67 and p_H· 3·3 to be accounted for. We have now to see what is the effect of temperature on the other amino-acids. The only data I have been able to find in the literature are those of Lundén [1906]. He determined the acid and basic dissociation constants of three simple amino-acids at 25° and 40°. I have calculated the shift of I.P. in each case, by means of the equation $R = \frac{I^2}{k_w}$ [Michaelis and Rona, 1910].

Ampholyte	Temperature	k_a	k_b	$\frac{k_a}{k_b} = R$	Iso-electric Point $R = \frac{I^2}{k_w}$ p_H·	k_w at 25° $= 10^{-14}$ at 40° $= 10^{-13\cdot49}$
Acetoxime	25°	$6\cdot0 \times 10^{-13}$	$6\cdot5 \times 10^{-13}$	0·92	7·02	
	40°	$9\cdot9 \times 10^{-13}$	$19\cdot0 \times 10^{-13}$	0·52	6·89	
β-i-Asparagine	25°	$1\cdot35 \times 10^{-9}$	$1\cdot53 \times 10^{-12}$	882·4	5·53	
	40°	$3\cdot22 \times 10^{-9}$	$4\cdot23 \times 10^{-12}$	761·2	5·56	
o-Amino-benzoic acid	25°	$1\cdot06 \times 10^{-5}$	$1\cdot38 \times 10^{-12}$	$7\cdot68 \times 10^{6}$	3·56	
	40°	$1\cdot35 \times 10^{-5}$	$3\cdot16 \times 10^{-12}$	$4\cdot37 \times 10^{6}$	3·68	

The basic constant in each case increases more than the acidic with rise of temperature, which leads to a displacement of the I.P. towards the alkaline side. In the case of acetoxime the basic constant is greater than the acidic and the I.P. is consequently on the alkaline side of neutrality, but in the last two the I.P. is on the acid side. Allowing again for the fact that the values of I.P. need correction as they are determined at different temperatures, which correction as we have already seen still further increases the difference, we find that the nett result of a rise of temperature on the I.P. in the case of ampholytes like β-i-asparagine and o-amino-benzoic acid, is to shift it towards the neutral point. This is exactly what has taken place with the lysin. It is true that the difference in the last case is much greater than

the more extended range of temperature would account for and it is quite possible that there are other factors concerned. It must be remembered, for instance, that the salt content of the lysin solution in the heat destruction experiments was very different from that in the cataphoresis experiments. However the lysin is in all probability much more complicated than such simple ampholytes as the ones cited, so that it is unwise to expect too close a parallel. From this point of view therefore the experimental facts are in harmony with the theory that the lysin is an ampholyte.

We have still to consider the effect of the presence of other substances on the behaviour of the lysin. I have already drawn attention to the fact that it is not pure. In biochemistry we are constantly faced with the difficulty that the body we are investigating is mixed with other substances which may influence its action. There is some experimental evidence bearing on this point. Firstly there are some anomalous instances of heat destruction of haemolysins. Madsen, Famulener, and Walbum [1904] found that the haemolysin of staphylococcus was greatly reduced in strength at 70°, but almost completely returned on raising the temperature to 100° for five minutes. Landsteiner and Rauchenbichler [1909] found the same thing and attributed it to a union of staphylolysin at a medium temperature with some other substance in the solution, which compound was dissociated again at 100°. Working with megatheriolysin I was able to show [Atkin, 1910] that the velocity of destruction increased up to a temperature of about 55°, then it decreased up to a temperature of 75°, after which it increased up to 100°. This phenomenon is probably due to the same cause as that operating in the case of staphylolysin. I also repeated Madsen and Famulener's experiments without being able to confirm their results—the staphylolysin showed only a gradually increasing destruction with rise of temperature. This seems further to substantiate the view that the return of activity at a high temperature is not a characteristic of the lysin itself, but due to the influence of some foreign substance. Naturally in different samples the quantitative if not the qualitative relations of the several constituents will vary. No such property exists in the case of nasik haemolysin however, possibly because of its relatively great thermolability, for it is completely destroyed at a temperature considerably less than that at which the supposed union takes place in the case of staphylolysin and megatheriolysin. Similar considerations also apply to tetanolysin. If the hypothesis that megatheriolysin undergoes a union with some other body at certain temperatures, which modifies its destruction by heat, is correct, the process of destruction

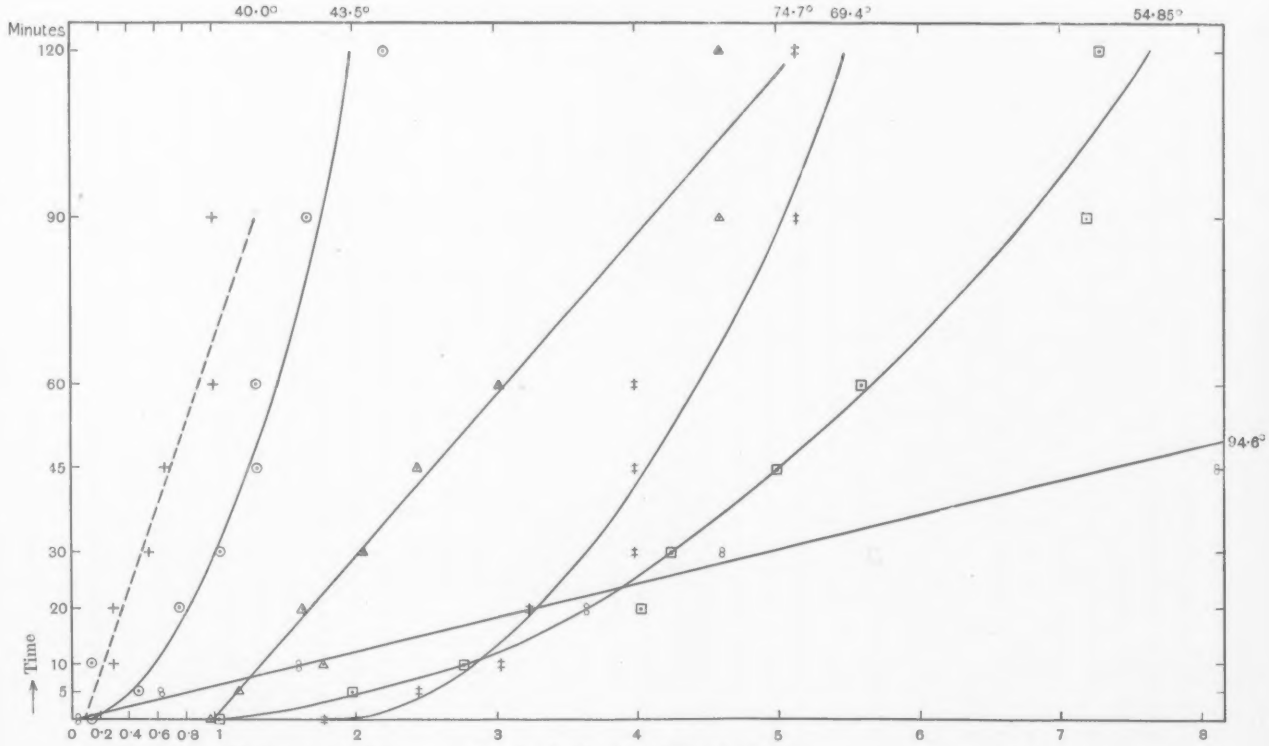


Fig. 2. Destruction of Megatheriolysin.

The points of origin of the curves are arbitrarily chosen for convenience of presentation.

should not be a unimolecular one. In other words, if we plot the logarithms of lysin concentration against time we ought not to get a straight line. If, on the other hand, at higher or lower temperatures such a combination either does not take place or is disrupted, it is reasonable to expect the process to be then a logarithmic one. I have applied this test to my experimental results in the case of megatheriolysin [Atkin, 1910] and find it actually to be so.

As the curves show (Fig. 2) the process of destruction is only unimolecular, firstly from a temperature of about 74°, upwards, which is near the point at which the rate of destruction again begins to increase with temperature; secondly, below a temperature of about 43°. It is not quite certain that the destruction at 40·0° is logarithmic, but at any rate it approximates closely to a straight line, if it is in reality a curve. It has been drawn in the figure as a dotted line. This last point is interesting as it shows that the influence of the foreign substance is being felt at a temperature considerably lower than that at which it is able to decrease the velocity of destruction with rising temperature. It will be noticed that the hypothetical union with another substance is not a reversible process, otherwise the decomposition which presumably takes place at high temperatures would result in a re-union on cooling down, for we must remember that the actual test on red blood cells is carried out at 37°. In fact the observation that we are able to detect the phenomenon at all renders it highly probable that it is irreversible, for if this were not so we should expect the union at medium temperatures to be split up again on cooling to 37°. We seem to have a criterion therefore with regard to the abnormal destruction of lysins by heat, namely, that if at any temperature it can be shown that the destruction is *not* unimolecular, the velocity of destruction will not rise regularly with temperature, or what is another phase of the same phenomenon, the strength of the lysin will decrease at a certain temperature, and reappear again at a higher one.

In my present experiments with nasik lysin the temperature was kept constant at 46°, the reaction alone being changed. It is hardly to be expected that a union with another body would take place having the same effect as in the case of megatheriolysin, seeing that this effect requires a considerably higher temperature, but nevertheless I have subjected the figures relating to the velocity of destruction to the same test and find that in every case the process is unimolecular. This fact is in favour of the chemical freedom of nasik lysin, under the conditions of these experiments.

Work bearing on the influence of impurities on the movements of enzymes in an electric field has been carried out by Pekelharing and Ringer [1911]. One of them devised a method of purifying crude pepsin by dialysing and thus reducing the acidity, so that the enzyme partially separates out. They do not claim that their product is absolutely pure, but at any rate it is more nearly pure than any commercial preparation. With it they obtained migration to the anode only, within the limits of acidity they tried. Addition of caseinogen, crystallised serum albumin, or albumose (Witte's peptone), caused migration more or less to the cathode. The pepsin was in fact carried by the albumin, etc. They were of opinion that the value $5{\cdot}5 \times 10^{-3}$ found by Michaelis and Davidsohn [1910] for the I.P. of pepsin was not reliable.

In the present case the lysin is mixed with, at any rate, one other protein substance which flocks out at a definite [H$^{\cdot}$]. It is difficult to say whether the lysin also flocks out at its I.P. as its absolute concentration must be extremely small and at 10° the I.P. apparently nearly coincides with that of the protein impurity. A glance at Table II, No. 4, shows that at this point there is no movement of the visible particles and at the same time the solution has become depleted of most of its lysin. We may here have a condition of things where the lysin and visible precipitate have opposite electric charges and consequently a union has taken place. The lysin would then presumably move with the precipitate. If this were so this boundary of the I.P. or rather isoelectric zone of the lysin would be rendered obscure. When however the sign of the charge of the lysin changed, the two bodies would again have similar charges and the lysin would be free to move independently. Similar considerations apply to the other boundary because the visible particles may become negatively charged before the lysin. A rough approximation to an isoelectric zone is therefore all that can be attained. If we take this zone as p_{H} 3·0 to 3·6 and assume that the geometric mean represents the I.P. then this will be the arithmetic mean of the p_{H} values, namely p_{H} 3·3. Therefore approximately,

Isoelectric point at 10° p_{H} 3·3.
" " 46° p_{H} 6·1.

This represents a large decrease in acid character with rise of temperature. It is interesting to attempt a determination of the acid and basic dissociation constants. Michaelis and Menten [1913] have devised a graphic method of obtaining these constants. Using the velocity constants I have constructed

dissociation-residue curves but find that they do not correspond with any theoretical curve, the steepness being much greater. The action of the H· and OH′ cannot be one of simple control of the dissociation of the lysin, but they must also take part in the destruction by heat. It is impossible therefore from the data to determine the dissociation constants, but the I.P., where H· and OH′ are in equal numbers, gives us a measure of the ratio of acid to basic constant.

From this investigation we can conclude that the experiments relating to heat destruction are in favour of the view that nasik haemolysin is an ampholyte. This is confirmed by the cataphoresis experiments. I must admit, however, that the presence of other proteins may influence the results, at any rate of the cataphoresis experiments. Similar objections may be raised to the whole series of the many determinations of the direction of movement in an electric field in the case of ferments, which have been investigated in the last few years, and it is quite possible that all these results will have to be revised.

Summary.

1. The velocity of destruction of nasik haemolysin at 46·0° is at a minimum slightly on the acid side of the neutral point. This is in favour of the view that the lysin is an ampholyte with its acid dissociation constant greater than its basic, and the point of minimum destruction is the isoelectric point. The anion and cation are therefore thermolabile, and the undissociated molecules not so.

2. Cataphoresis experiments at 10° confirm this view.

3. Approximate values for the isoelectric points are

$$p_{H\cdot} \text{ at } 10^\circ = 3{\cdot}3\,; \quad p_{H\cdot} \text{ at } 46^\circ = 6{\cdot}1.$$

This decrease of acidity with rising temperature is also found in the case of simple ampholytes, but to a smaller extent.

4. Incidentally a criterion is given for the anomalous heat destruction of certain haemolysins, for instance, megatheriolysin, namely, that the process is not unimolecular at temperatures where the velocity of destruction decreases with rising temperature, but it can be shown to be so at temperatures considerably removed where the velocity of destruction follows the more ordinary course of increase with rise of temperature.

REFERENCES.

Atkin (1910). *Zeitsch. Immunitätsforsch.*, **7**, 656.
Bechhold (1907). *München. med. Woch.*, **54**, 1921.
Bjerrum (1905). *Zeitsch. physikal. Chem.*, **53**, 428.
Famulener and Madsen (1908). *Biochem. Zeitsch.* **11**, 186.
Field and Teague (1907). *J. Exp. Med.*, **9**, 86.
Kraus and Levaditi (1908). *Handbuch d. Techn. u. Method. d. Immunitätsforschung*, **1**, 57.
Landsteiner (1913). Article on *Kolloide und Lipoide in der Immunitätslehre.* Kolle and Wassermann's *Handbuch der pathogenen Mikroorganismen.*
Landsteiner and Pauli (1908). *Verhandl. 25th Kongr. f. Innere Med. Wien.*, 571.
Landsteiner and Rauchenbichler (1909). *Zeitsch. Immunitätsforsch.*, **1**, 439.
Lundén (1906). *Zeitsch. physikal. Chem.*, **54**, 532.
— (1908). *Affinitätsmessungen an Schwachen Säuren und Basen*, 45. Bd. XIV aus der Sammlung chemischer und chemisch-technischer Vorträge. Ferdinand Enke. Stuttgart.
Madsen, Famulener and Walbum (1904). *British Med. J.*, **2**, 572.
Michaelis and Davidsohn (1911). *Biochem. Zeitsch.*, **36**, 280.
— (1910). *Biochem. Zeitsch.*, **28**, 1.
Michaelis and Menten (1913). *Biochem. Zeitsch.*, **49**, 333.
Michaelis and Rona (1909). *Biochem. Zeitsch.*, **18**, 317.
— (1910). *Biochem. Zeitsch.*, **27**, 38.
Noyes, A.A., and others (1910). *Zeitsch. physikal. Chem.*, **70**, 335.
Pekelharing and Ringer (1911). *Zeitsch. physiol. Chem.*, **75**, 282.
Sörensen (1912). *Ergebnisse d. Physiol.*, **12**, 393.
Teague and Buxton (1907). *J. Exp. Med.*, **9**, 254.

XL. THE RELATIONS OF VITAMINE TO LIPOIDS.

By EVELYN ASHLEY COOPER, *Beit Memorial Research Fellow, Lister Institute.*

(*Received July 8th*, 1914.)

Although vitamine, i.e. the substance which prevents beri-beri and avian polyneuritis, has been shown to be present in higher concentration in various tissues rich in lipoid, e.g. liver, cardiac muscle, brain (ox) and egg-yolk, than in voluntary muscle (ox, fish) which is poor in lipoid [Cooper, 1913, 1] it is now generally believed that the active substance as isolated from foodstuffs is not a lipoid. It contains no phosphorus or carbohydrate [Cooper and Funk, 1911; Funk, 1911, 1914; Edie, Evans, Moore, Simpson, Webster, 1912] and is therefore neither a phosphatide nor cerebroside. MacLean [1912] furthermore showed that lecithin, which had previously been found by Schaumann [1910] to be slightly curative, had still less effect after purification, and he concluded that the anti-neuritic substance is not a lipoid but is present in lipoids as an impurity extractable by simple methods. Cooper [1913, 2] subsequently found that when the fats and lipoids (alcoholic extract) of horse voluntary muscle were treated with ether the bulk of the anti-neuritic substance contained therein was precipitated and by a complex fractionation of the active precipitate a substance was isolated, small doses of which readily cured polyneuritis in pigeons. 4 g. of the original alcoholic extract exerted a rapid curative action in neuritic pigeons, but after one extraction with ether as much as 12 g. of the fats and lipoids (ether-soluble fraction) had only a slight ameliorative effect. More than 66 per cent. of the total amount of active material present in the original extract could thus be removed by simple treatment with ether and was thus apparently not in chemical union with the lipoids. Similar results were obtained when the flesh was not air-dried at 30° before extraction with alcohol [Cooper, 1913, 1]. This showed that the anti-neuritic substance was not split off from a lipoid complex during the process of air drying.

The above facts however do not exclude the possibility that, although

the anti-neuritic substance is preformed in the diet, in the nervous system it in part becomes chemically combined with other substances to form a lipoid.

Experiments on similar lines have therefore been carried out with cardiac muscle and brain, and the results obtained are set forth in the present communication. The work with heart muscle was undertaken primarily with the object of isolating a sufficient amount of the anti-neuritic substance for a study of its chemical nature, the cardiac muscle being substituted for voluntary muscle on account of its higher content of active material [Cooper, 1913, 1].

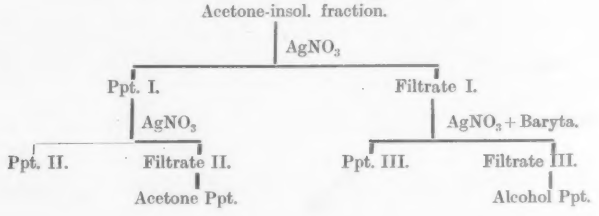
It must first be pointed out however that according to Vedder and Williams [1913] dry and wet beri-beri are caused by a deficiency in the dietary of distinct substances. In birds as a rule only dry beri-beri occurs, and, although infantile beri-beri is almost exclusively of the wet type, no signs of this condition were observed by the author even in very young chickens fed on polished rice. In this paper we are therefore only concerned with the substance which prevents and cures dry beri-beri and avian polyneuritis.

I. Preparation of an anti-neuritic substance from cardiac muscle.

150 lbs. of ox-heart were minced, dried at 30° by means of an electric fan, ground, and thoroughly extracted with 95 % alcohol. The filtered extract, from which the alcohol was removed by evaporation *in vacuo*, was treated with an excess of ether, and the material precipitated possessed marked anti-neuritic properties, 0·2 g. given orally rapidly curing pigeons affected with polyneuritis. The procedure subsequently followed was a modification of that employed in the fractionation of horse-flesh and is briefly described below.

The ether-insoluble precipitate was taken up with 95 % alcohol which dissolved a considerable amount of material. The insoluble residue contained both inorganic and organic matter, but was inactive. The soluble fraction however was strongly curative and was next treated with excess of ether, which completely precipitated the anti-neuritic substance. The precipitate was dissolved in water and allowed to stand in a vacuum desiccator for several days. A white crystalline substance slowly separated, but this had no curative action. The supernatant liquor was then treated with basic lead acetate until no more precipitate was formed. As in the case of horse-flesh, the active substance was not precipitated by this reagent, and the filtrate

possessed marked curative properties. So far the method was identical with that employed in the case of horse-flesh, but at this stage a departure from the original process was made. It was found in the previous work that the addition of silver nitrate after the lead acetate precipitation only partially precipitated the curative substance. This manipulation was therefore postponed in the subsequent technique with the object of ascertaining what influence intermediate fractionations might have upon the precipitability of the substance by the silver salt. The lead acetate filtrate after the removal of excess of lead by dilute sulphuric acid was therefore evaporated *in vacuo* and the concentrated solution treated with excess of 95 per cent. alcohol. By this procedure some material was separated, but all the anti-neuritic substance remained in solution. The filtrate was evaporated to a syrup and treated with acetone, which precipitated practically all the active substance. The filtrate possessed only slight curative properties, whilst 0·15 g. of the precipitated substance given orally rapidly cured pigeons affected with polyneuritis. The substance was insoluble in benzene, ether, and chloroform, but dissolved readily in water. The aqueous solution was next treated with silver nitrate solution, which however still only precipitated a portion of the active substance, and the latter could furthermore only be partially precipitated from the filtrate by the addition of baryta. The first precipitate (with silver nitrate only) was decomposed with dilute hydrochloric acid and silver nitrate again added to the filtered solution. On this occasion the substance was not precipitated to any appreciable degree and the filtrate was strongly curative. The filtrate after removal of excess of silver as the chloride was concentrated *in vacuo* and was then treated with acetone. A substance was thereby precipitated, 0·04 g. of which given orally quickly cured polyneuritis in pigeons whilst 0·008 g. cured by subcutaneous injection. The filtrate from the third precipitate (obtained with silver nitrate and baryta) was concentrated and treated with 95 per cent. alcohol, and a substance was precipitated, 0·04 g. of which given orally also cured polyneuritis. The manipulations are set forth schematically below.



(The thick lines represent the main course of the active substance during the fractionations.)

The substances separated in the above way from filtrates II and III and the solution obtained by decomposition of precipitate III were now combined and treated with 95 per cent. alcohol, which precipitated a small amount of material possessing no curative properties. The filtrate was evaporated and acetone added. This caused the precipitation of a substance (5 g.) 0·04 g. of which administered orally readily cured neuritic pigeons. The filtrate was only slightly curative.

On standing a few days in a sulphuric acid desiccator the substance however ceased to have any curative action, and even large doses only prolonged the lives of neuritic birds. It was thus of little interest to carry out a chemical analysis of the substance.

It is evident therefore that the anti-neuritic substance is extremely labile and with the chemical methods at present at our disposal its isolation will prove a very difficult matter. Further work must be carried out to ascertain the conditions favourable to the existence of the active substance. For this reason and also on account of cost it is doubtful if highly purified preparations will be of much therapeutical value.

An inexpensive method however of preparing a non-toxic highly curative solution suitable for use in the treatment of human beri-beri was described in a previous communication [Cooper, 1914, 2].

II. The relations of Vitamine to Lipoids.

The curative power of the original alcoholic extract of cardiac muscle was determined before and after treatment with ether. It was found that about 3 g. of the extract readily cured polyneuritis in pigeons, but after the extract was treated with ether 4 to 8 g. of the fats and lipoids (ether-soluble fraction) had little or no curative action. The small amount of active substance still retained by them could not be removed by a second extraction with ether, but by treating the ether-soluble fraction with acetone the phosphatides were precipitated and as much as 10 g. had not the slightest effect upon neuritic pigeons. These observations support the conclusion of MacLean (p. 347).

Different observations however were made in the case of brain. 13 kilos. of ox-brain were minced, dried at 30° for 16 hours, and extracted with alcohol on the shaking machine. The filtered extract was concentrated

in vacuo to remove the alcohol, and when dry weighed 730 g. The extract was next treated with excess of ether, which precipitated 100 g. of a white substance. 0·6 g. of this rapidly cured polyneuritis in pigeons, but 0·4 g. had only a slight action.

This fraction from brain was less curative than the corresponding fractions from voluntary muscle and cardiac muscle, 0·2 g. of which was quite sufficient to bring about recovery. It also differed from the voluntary muscle preparations in not being completely soluble in water. The ether-soluble fraction from brain, on the other hand, unlike the analogous fractions from muscle, possessed marked curative properties, 2-3 g. being sufficient to cure pigeons affected with polyneuritis. The fats and lipoids still retained their activity even when extracted three times with ether.

On treating the ether-soluble fraction with acetone however the active substances were present in the acetone-soluble fraction, 1–3 g. of which readily cured, and, as was observed in the case of cardiac muscle, the precipitated phosphatides in doses of 5 to 10 g. possessed no detectable curative power. The phosphatides had no curative effect even after hydrolysis with 5 per cent. sulphuric acid, a process which does not affect the active substance. This shows that the negative results were not due to the indigestibility of the lipoids.

Similar results were obtained when alcoholic extracts of sheep-brain and egg-yolk, were treated with ether. There would thus appear to be present in brain and egg-yolk an ether- and acetone-soluble substance which possesses a marked affinity, chemical or adsorptive, for vitamine, and which may function as a carrier in the living organism.

The residues left after extracting the ox- and sheep-brain with alcohol were retained, and experiments carried out to ascertain to what extent the anti-neuritic substance contained in brain was extracted by alcohol.

13 kilos. of the ox-brain yielded 1680 g. of residue after exhaustion with alcohol. It was previously found [Cooper, 1914, 1] that the addition of 6 g. of ox-brain to the polished rice diet (1/20th body-weight ration daily) was sufficient to prevent polyneuritis in pigeons for at least 50 days.

Two pigeons were therefore fed daily on the above ration of polished rice and an amount of the brain residue equivalent to 12 g. of brain (1·5 g.). Both birds developed polyneuritis in 30 days. It is evident from this result that the greater part of the anti-neuritic substance contained in ox-brain is extracted by 95 per cent. alcohol.

1900 g. of the sheep-brain yielded 238 g. of alcohol-insoluble material.

It was previously observed [Cooper, 1913, 1] that the addition of about 8 g. of sheep-brain to a 1/20th body-weight ration of polished rice daily prevented polyneuritis in pigeons for 50 days. Three pigeons were therefore fed daily on the above ration of rice and an amount of the brain residue equivalent to 15 g. of the original brain (2 g.). All the birds developed polyneuritis within 30 days. As in the case of ox-brain, the bulk of the anti-neuritic substance is evidently extracted along with the fats and lipoids by 95 per cent. alcohol.

It having been demonstrated that brain phosphatides when purified possessed no curative power, experiments were next carried out to ascertain if the anti-neuritic substance were more firmly combined with certain other constituents of brain. Cholesterol prepared by crystallisation from an acetone extract of brain was first tested on neuritic pigeons, but 1 g. was found to exert no curative action.

1 kilo. of ox-brain was next minced and without drying repeatedly extracted with absolute alcohol at 40°. The extract was filtered and kept at 0° for 12 hours. By that time a yellowish precipitate (protagon) separated out. This was filtered off and washed with cold alcohol, the residue weighing 15 g. 2 to 5 g. of this deposit however had no effect at all upon neuritic pigeons.

5 g. were then treated with ether. The insoluble residue (3 g.) containing cerebroside (cerebrone) had no curative action, and kephalin (1 g.) precipitated from the ethereal extract by the addition of absolute alcohol also proved to be inactive. The original alcoholic extract after removal of the " protagon " was concentrated *in vacuo*, and the residue treated with ether. As before, the precipitate was curative. It was extracted with absolute alcohol at 0°, and the undissolved substance (1·5 g.) containing cerebrosides was again inactive. The alcohol-soluble fraction was concentrated *in vacuo* and when dry weighed 5 g. This was highly curative, 0·25 to 0·5 g. being adequate to cure polyneuritis.

The original ether-soluble fraction containing the phosphatides and fats of the brain was freed from ether and treated with acetone. The precipitated phosphatides were dissolved in a little chloroform and again reprecipitated with acetone. The phosphatides in doses of from 4 to 8 g. were inactive but 1 to 2·5 g. of the acetone-soluble fraction readily cured pigeons, thus confirming the observations detailed earlier in the paper.

This work was then repeated on a larger scale, 3 kilos. of ox-brain being used. Similar results were obtained, protagon (even when hydrolysed)

kephalin, cerebrone, and the phosphatides again being found to be ineffective when given to neuritic pigeons in approximately the above mentioned doses.

The failure of protagon, kephalin, cerebrone, cholesterol and phosphatides to cure polyneuritis is of interest in relation to the researches of Stepp [1909, 1911, 1913] who has found that food extracted with alcohol-ether is deleterious to the health of mice, and that the harmful effect of the diet is not checked by the addition of lecithin, cholesterol, kephalin, cerebrone, and phytin. When however egg-yolk or alcoholic extracts of egg-yolk or brain are added the animals remain healthy. Of these substances cholesterol, kephalin, and cerebrone as stated above do not contain vitamine, and lecithin [MacLean, 1912; Cooper, 1913, 2] and phytin [Schaumann, 1910; Cooper and Funk, 1911] when purified are also practically free from the active substance.

Egg-yolk and alcoholic extracts thereof on the other hand contain the essential substance [Cooper, 1913, 1], as also do alcoholic extracts of brain [Funk, 1912; Cooper, 1913, 2]. It is therefore probable that the harmful effects of diets consisting of food extracted with alcohol-ether is due not to the deficiency in the diets of lipoids, but to a deficiency of vitamine which is extracted by alcohol along with the lipoids.

The main conclusion that emerges from these observations is that vitamine does not apparently enter into the constitution of the lipoids of the brain, but may possibly be stored therein until required in the nutrition of the nervous system.

To what extent lipoids can adsorb the active substance from an *aqueous* medium is a point to be settled by further experiment. Meanwhile it may be noted that asbestos (Ural) does not appreciably adsorb vitamine from solutions of autolysed yeast (acid reaction).

Summary.

1. Although infantile beri-beri is almost exclusively of the wet type, young chickens fed on polished rice develop symptoms of the dry form only, like adult birds.

2. A method is described for the preparation from cardiac muscle of a substance small amounts of which cure polyneuritis in pigeons. The substance however was extremely unstable and lost its activity in a few days after isolation.

3. As in the case of voluntary muscle, the bulk of the anti-neuritic

substance contained in the fats and lipoids (alcoholic extract) of cardiac muscle can be separated therefrom by means of ether.

4. In the case of brain, however, the anti-neuritic substance can only be partially precipitated from the alcoholic extract by treatment with ether, but can be entirely removed from the phosphatides by the subsequent addition of acetone. An explanation of this is offered.

5. Purified brain phosphatides possess no curative power even after hydrolysis with acid. This shows that their inability to cure polyneuritis is not due to incomplete digestion.

6. Protagon (even when hydrolysed), kephalin, cholesterol, and cerebrone similarly have no curative action.

7. The bulk of the anti-neuritic substance contained in sheep- and ox-brain can be extracted by 95 per cent. alcohol.

Conclusion.

The results show that vitamine does not enter into the constitution of the lipoids of brain and muscle, but may possibly be adsorbed in the lipoids and so stored, until required by the organism.

It would thus appear that the deleterious effect of lipoid-free diets observed by Stepp is due not to the deficiency of lipoids, but to the mechanical removal of vitamine during the alcohol-ether extractions.

I desire to express my best thanks to Dr Hugh MacLean for kindly help in the course of this investigation.

REFERENCES.

Cooper (1913, 1). *J. Hyg.*, **12**, 436.
— (1913, 2). *Biochem. J.*, **7**, 268.
— (1914, 1). *J. Hyg.*, **14**, 11.
— (1914, 2). *Biochem. J.*, **8**, 250.
Cooper and Funk (1911). *Lancet*, **2**, 1266.
Edie, Evans, Moore, Simpson, Webster, (1912). *Biochem. J.*, **6**, 234.
Funk (1911). *J. Physiol.*, **43**, 395.
— (1912). *J. Physiol.*, **45**, 75.
— (1914). *J. Physiol.*, **48**, 228.
MacLean (1912). *Biochem. J.*, **6**, 355.
Schaumann (1910). *Arch. Schiffs. Tropenhyg.*, **14**, 8.
Stepp (1909). *Biochem. Z.*, **22**, 452.
— (1911) *Zeitsch. Biol.*, **57**, 135.
— (1913). *Zeitsch. Biol.*, **62**, 405.
Vedder and Williams (1913). *Philippine J. Science*, **8**, 175.

XLI. THE RELATIVE AMOUNTS OF β-HYDROXYBUTYRIC ACID AND ACETO-ACETIC ACID EXCRETED IN ACETONURIA.

By ERNEST LAURENCE KENNAWAY.

From the Second Medical Clinic, Munich, and the Physiological Laboratory, Guy's Hospital.

(*Received July 9th*, 1914.)

Various workers have shown that a reversible reaction occurs in the body between aceto-acetic acid and β-hydroxybutyric acid [for references see Dakin, 1912]

$$\underset{+O}{CH_3 . CHOH . CH_2 . COOH} \rightleftarrows \underset{+H_2O}{CH_3 . CO . CH_2 . COOH.}$$

Neubauer [1910] showed that when aceto-acetic acid was administered to a diabetic, not only this acid, but also β-hydroxybutyric acid was excreted in increased amounts, and he confirmed the older statement that the administration of β-hydroxybutyric acid in diabetes increased the output of both acids. He pointed out further that the relative amounts of the two acids in the urine in cases of acetonuria should indicate the equilibrium point of the reversible reaction within the body. He found that the amount of β-hydroxybutyric acid, expressed as a percentage of the total acetone bodies, was remarkably constant, this percentage being from 60 to 80 in well-marked cases of acetonuria, and somewhat lower than this when the total output of acetone bodies was small. In spite of the enormous numbers of estimations of acetone bodies which have been made, no other writers appear to have paid much attention to the relative amounts of these substances. The cases given below serve to provide some illustrations of Neubauer's statement.

It is necessary to have some short term by which to express the relative amounts of β-hydroxybutyric acid and aceto-acetic acid in urine. The term

" β-ratio " is used below to denote the percentage of the total acetone bodies which is made up by β-hydroxybutyric acid, each substance being calculated in the form of acetone.

No attempt is made here to establish with any great accuracy the proportions in which the different acetone bodies are produced in the body. To do this in a satisfactory manner, one would require firstly an accurate method for the estimation of β-hydroxybutyric acid. The method used in nearly all these observations gives results which are from 4 to 10 % too low [Shaffer and Marriot, 1913; Kennaway, 1914]. If the β-ratio in a urine were actually 75, and the result of the β-hydroxybutyric acid estimation were 10 % too low, the β-ratio found would be 72·96. Secondly, one would require estimations of the amount of acetone in the expired air. Since there is a considerable fall in molecular weight in the formation of acetone (from 102 to 58), the comparatively small amounts of this substance in the breath are of importance in ascertaining the total production of acetone bodies.

The estimations on Case 1 below were made by the Messinger-Huppert and polarimetric methods, and all the others by a combination of the Shaffer and Scott-Wilson methods [Kennaway, 1914]. The pathological urines were obtained with two exceptions from the wards of Guy's Hospital, and I wish to express my thanks for the material to the physicians in charge of these cases. I am greatly indebted also to Drs Pembrey, Poulton, and Graham for permission to include in the tables their estimations of alveolar carbon dioxide, and to Drs Poulton and Graham for the opportunity to make observations in the course of their numerous experiments upon themselves [Graham and Poulton, 1913, 1914, 1, 2].

Cases of Diabetic Acidosis.

If a person is excreting two molecules of β-hydroxybutyric to one of aceto-acetic acid, the β-ratio will be 66·6; if five molecules to one, it will be 83·3, and so on. In Fig. 1, all the β-ratios obtained from Cases 1–14 of diabetes are arranged in order of magnitude; Case 15, which showed a much smaller output of acetone bodies and lower β-ratio than any of the others, is omitted to save space (see Table IV).

It is hardly necessary to point out that this figure shows merely the range observed in these cases; it does not of course show the frequency of occurrence of any particular ratio, the number of points near the level of 75 being due to the number of estimations made on Case 4.

The results given in the tables below indicate that Case 1 was excreting chiefly two molecules of β-hydroxybutyric acid to one of aceto-acetic acid; in Case 4, the proportion was about three to one, and in Case 7 probably five to one.

Fig. 1. β-ratios of Cases 1–14 (Diabetes), in order of magnitude. × indicates urine at autopsy.

TABLE I.

Cases 1-3. *Diabetes.*

	Date	Urine c.c.	G. acetone per day: Aceto-acetic acid + acetone	G. acetone per day: β-hydroxy-butyric acid	G. acetone per day: Total	β-ratio
Case 1.	27 vii.	1650	1·29	3·22	4·51	71·4
	29 vii.	2460	1·01	2·056	3·066	67 1
	30 vii.	1700	0·51	1·037	1·547	67·0
	1 viii.	1500	0·472	0·975	1·447	67·4
	2 viii.	1210	0·454	0·970	1·424	68·0
Case 2.	28 i.	3380	5·24	14·13	19·37	73·0
	6 ii.	5000	6·41	20·85	27·26	76·5
Case 3.	1 v.	4145	5·43	20·67	26·10	79·2
	7 v.	4125	6·04	21·55	27·59	78·1
	9 v.	3030	4·85	19·37	24·22	80·0

Case 4 (Table II and Fig. 2) had received before coming under observation large doses of sodium bicarbonate, on account of signs of incipient coma. The alveolar carbon dioxide pressure was found by Dr Pembrey to be abnormally high (49·3 mm.), no doubt as a result of the ingestion of alkali. In the table, the doses of bicarbonate are calculated as acetone derived from the

amounts of β-hydroxybutyric acid which could be neutralised by the bicarbonate, in order to show the assistance given to the neutralising powers of the body. On two occasions (Aug. 9th and 18th) the dose of alkali was doubled, and the alveolar carbon dioxide pressure showed a slight increase.

TABLE II.

Case 4. *Diabetes.*

Date	Urine c.c.	Alveolar CO_2 mms. Hg.	$NaHCO_3$ as g. acetone per day	G. acetone per day: Aceto-acetic acid + acetone	G. acetone per day: β-hydroxy-butyric acid	G. acetone per day: Total	β-ratio
18 vii. ..	—	—	62	—	—	—	—
19	—	—	92	—	—	—	—
20 ..	—	—	32	—	—	—	—
21 ..	4640	49·3	16	6·39	14·72	21·11	69·8
22 ..	4000	46·4	,,	5·37	13·19	18·56	71·1
23 ..	4040	39·1	,,	3·99	10·68	14·67	72·8
25 ..	4250	39·4	,,	3·59	12·01	15·60	76·9
28 ..	5830	35·7	,,	4·54	13·71	18·25	75·1
30 ..	6670	34·2	,,	6·57	19·21	25·78	74·5
31 ..	6510	—	,,	7·82	21·24	29·06	73·1
1 viii. ..	4560	33·3	,,	4·96	13·28	18·24	72·8
3 ..	2710	31·4	,,	3·96	11·86	15·82	75·4
6 ..	4270	33·9	,,	4·00	12·26	16·26	75·4
7 ..	4600	—	,,	4·15	13·96	18·11	77·1
8 ..	3130	36·0	,,	1·94	5·99	7·93	75·6
9 ..	7390	40·3	32	5·36	16·84	22·20	75·9
10 ..	4810	—	32	3·56	9·58	13·14	72·9
11 ..	9290	37·8	16	4·85	12·20	17·05	71·5
12 ..	7790	38·1	,,	6·87	21·96	28·83	76·2
13 ..	6770	33·3	,,	5·82	18·25	24·07	75·8
17 ..	5820	—	,,	7·03	19·95	26·98	73·8
18 ..	7640	34·3	32	7·83	21·49	29·32	73·3
19 ..	9670	36·6	32	7·89	21·80	29·69	73·4
20 ..	7260	35·2	16	4·21	12·21	16·42	74·3
						mean 20·34	mean 74·1

In Cases 5–13, the urine present in the bladder at autopsy was examined; the results obtained are enclosed in brackets in the tables. The β-ratio in this urine is in most cases low, and in those in which the comparison is possible (Cases 5, 7, 8, 9)[1], it is lower than it was on the previous days; a similar change was observed in a non-diabetic condition (Case 26). The explanation of this is not clear. One would not expect the oxidative change of the

[1] In case 6 the comparison cannot be made owing to the week's interval after the last estimation before death.

reversible reaction to be favoured in the hours preceding death. Possibly a diminished output of acetone from the lung may be the cause of the difference.

TABLE III.

Cases 5-13. *Diabetes.*

	Date	Urine c.c.	Alveolar CO_2 mm. Hg.	G. acetone per day: Aceto-acetic acid + acetone	G. acetone per day: β-hydroxy-butyric acid	G. acetone per day: Total	β-ratio
Case 5. ..	25 vi.	1860	27·4	3·47	15·77	19·24	81·9
	2 vii.	2200	27·0	4·42	14·72	19·14	76·9
	3 vii.	2000	—	5·06	15·74	20·80	75·7
	4 vii.	1860	26·9	3·92	11·11	15·03	73·9
	5 vii.	3600	24·5	8·21	24·49	32·70	74·9
	7 vii.	—	17·6	—	—	—	—
Not 24 hours urine { Coma	8 vii.	—	8·8	—	—	—	71·0
"	9 vii.	—	—	—	—	—	70·0
Death on ..	9 vii.	—	—	—	—	—	—
Urine at autopsy ..	—	—	—	—	—	—	[63·4]
Case 6	3 iii.	3820	—	3·33	10·30	13·63	75·5
	4 iii.	3860	—	4·12	12·31	16·43	74·9
	5 iii.	3090	—	3·94	12·60	16·54	76·2
	10 iii.	5305	—	5·99	18·18	24·17	75·2
	11 iii.	4830	—	4·58	11·97	16·56	72·3
	12 iii.	6050	—	5·98	15·67	21·65	72·4
Death in coma on	20 iii.	—	—	—	—	—	—
Urine at autopsy ..	—	—	—	—	—	—	[77·6]
Case 7.	10 iii.	5580	13·8	4·30	20·33	24·63	82·5
Coma on ..	12 iii.	—	—	—	—	—	83·6
Death on ..	13 iii.	—	—	—	—	—	—
Urine at autopsy ..	—	—	—	—	—	—	[77·7]
Case 8.	30 v.	4800	29·7	10·56	23·66	34·22	69·1
Coma. Death on	2 vi.	—	—	—	—	—	—
Urine at autopsy ..	—	—	—	—	—	—	[68·4]
Case 9.	30 i.	—	17·8	—	—	—	70·3
No coma, death on	31 i.	—	—	—	—	—	—
Urine at autopsy ..	—	—	—	—	—	—	[68·1]
At autopsy, females: *Case* 10 ..	—	—	—	—	—	—	[69·7]
At autopsy, females: *Case* 11 ..	—	—	—	—	—	—	[66·6]
At autopsy, females: *Case* 12 ..	—	—	—	—	—	—	[66·2]
At autopsy, females: *Case* 13 ..	—	—	—	—	—	—	[67·4]

Cases 14 and 15 showed a much smaller output of acetone bodies, and the β-ratios are low. In Case 15, the urine was almost free from sugar; the amount of acetone from aceto-acetic acid decreased to 11 mg. per day, while normal urine yields from 2 to 4 mg. [Scott-Wilson, 1911].

TABLE IV.

Cases 14 *and* 15. *Diabetes.*

	Date	Urine c.c.	Alveolar CO_2 mm. Hg.	G. acetone per day: Aceto-acetic acid + acetone	G. acetone per day: β-hydroxy-butyric acid	G. acetone per day: Total	β-ratio
Case 14.	13 v.	1240	36·1	0·347	0·627	0·974	64·4
	20 v.	1350	35·2	0·226	0·278	0·504	55·1
Case 15.	7 v.	1425	—	0·103	0·087	0·190	45·8
	20 v.	1580	38·7	0·0114	0·0077	0·0191	40·2

The largest change in the β-ratio within three days observed in this series of diabetics is that from 71·5 to 76·2 in Case 4.

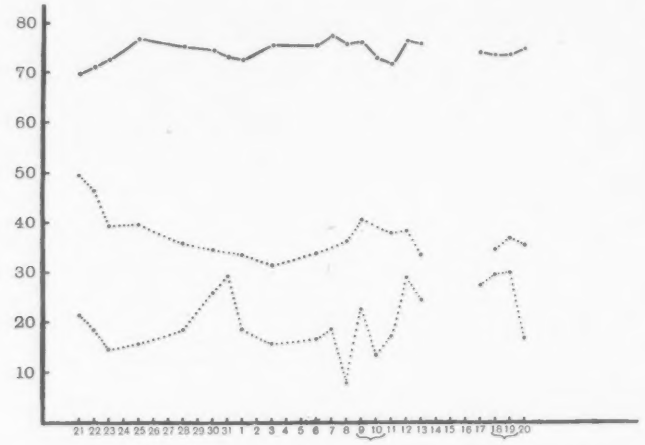


Fig. 2. Case 4 (Diabetes). Continuous line represents β-ratio. Upper dotted line represents alveolar CO_2 pressure in mm. Hg. Lower dotted line represents daily output of total acetone bodies as grams of acetone. The days on which the dose of sodium bicarbonate was doubled are enclosed in brackets.

Cases of Non-diabetic Acidosis.

In the first days of carbohydrate starvation in a healthy person no constancy of the β-ratio, such as is seen in cases of diabetes, is to be expected, since the degree of acidosis and the whole state of metabolism are changing

rapidly. The ratio rises with the increasing formation of acids, and may reach the value seen in cases of severe diabetes (Table V and Fig. 3).

TABLE V.

Acidosis due to lack of carbohydrate in healthy persons.

	Urine c.c.	Alveolar CO_2 mm. Hg	G. acetone per day: Aceto-acetic acid + acetone	G. acetone per day: β-hydroxy-butyric acid	G. acetone per day: Total	β-ratio
Case 16.						
Diet of protein and fat (Graham)						
2nd day	700	33·4	0·280	0·303	0·583	52·0
3rd day	1310	30·8	0·754	1·539	2·293	67·0
Case 17.						
Diet of protein and fat (Poulton)						
2nd day	670	38·3	0·402	0·521	0·923	56·4
3rd day	1070	37·0	0·446	0·556	1·002	55·4
Case 18.						
Diet of protein and fat (Donaldson)						
2nd day	758	36·5	0·437	0·299	0·736	40·7
Case 19.						
Starvation (Poulton)						
1st day	410	—	0·087	0·101	0·188	53·8
2nd day	1230	36·1	0 584	1·007	1·591	63·3
3rd day	730	35·3	0·539	1·383	1·922	72·0
Case 20.						
Starvation (Graham)						
2nd day	1240	36·1	0·477	0·692	1·169	59·2
3rd day	1680	33·0	0·527	0·905	1·432	63·2
Diet of fat						
4th day	1300	32·5	0·462	1·126	1·588	70·9
5th day	1390	—	0·882	2·638	3·520	74·9

Cases 21, 22, and 23 (Table VI) show low ratios with the excretion of very small amounts of acetone bodies. Some of the samples obtained from cases of vomiting (24 to 28) in young children were very similar as regards percentage and proportions of acetone bodies to the urine in severe forms of diabetes. Case 24 was subject to attacks of cyclical vomiting, in which condition well-marked acid intoxication occurs, but whether the illness on this occasion was of that nature was uncertain. Case 26 showed a condition of hyperpnoea and drowsiness such as is seen before the onset of coma in diabetes; the administration of dextrose and sodium bicarbonate had no beneficial effect, and the child died on the fifth day of the illness. The volume of urine was of course small as a result of the vomiting, and the percentages of acetone

bodies in this case are the highest in the whole series. Unfortunately the 24 hours output could not be ascertained. Cases 27 and 28 (Table VII) show

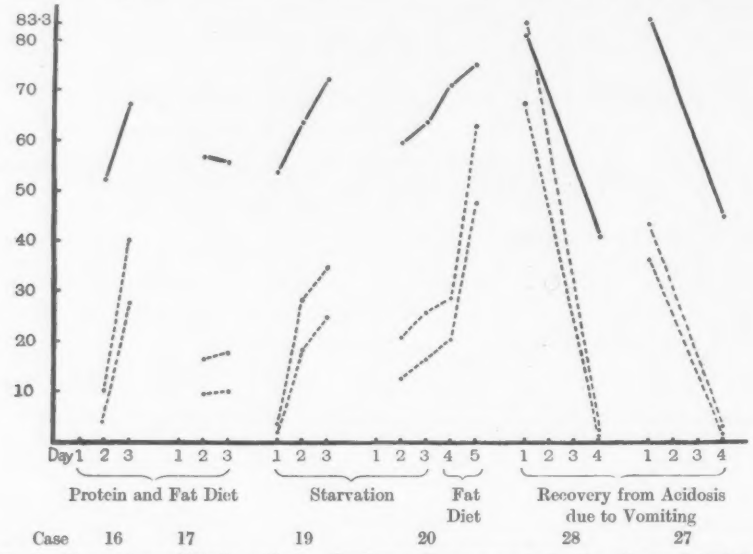


Fig. 3. Cases of non-diabetic acidosis. Continuous line represents β-ratio. Lower dotted line represents day's output of β-hydroxybutyric acid in decigrams. Upper dotted line represents day's output of β-hydroxybutyric acid + aceto-acetic acid in decigrams.

TABLE VI.

			G. acetone in 100 c.c. urine			
			Aceto-acetic acid + acetone	β-hydroxy-butyric acid	Total	β-ratio
Case 21. Carcinoma of Oesophagus						
after about 36 hours fasting			0·016	0·0094	0·0254	37·0
4 hours later			0·0147	0·0123	0·027	45·6
Case 22. After anaesthesia			0·0181	0·0083	0·0264	31·6
Case 23. „ „			0·054	0·077	0·131	58·8
Case 24 (age 2 years). Vomiting			0·058	0·149	0·207	71·5
Case 25 (age 5 years). „			0·139	0·468	0·607	77·1
Case 26 (age 22 months). „ .. 19 vi.			0·193	0·766	0·959	80·0
21 vi.			0·316	0·925	1·241	74·6
Death on 22 vi.			—	—	—	—
Urine at autopsy			0·357	0·956	1·313	[72·8]
		Urine c.c.	Amounts in 24 hours			
Case 27 (age 7 years). Vomiting 22 v.		520	0·393	2·033	2·42	83·8
Recovery .. .. .. 25 v.		550	0·086	0·069	0·155	44·6
27 v.		550	trace	trace	—	—
Case 28 (age 6 years). Vomiting 24 vi.		575	0·917	3·76	4·67	80·4
Recovery .. .. .. 27 vi.		1210	0·062	0·042	0·104	40·7

the fall in the β-ratio which occurs when the total amount of acetone bodies decreases (Fig. 3). The high proportion of β-hydroxybutyric acid in these cases is noteworthy. Case 28 excreted on one day 0·3 g. acetone bodies per kilo. body-weight (15·5 kilos.); in a patient with severe diabetes, excreting 35 g. acetone bodies and weighing 70 kilos., the output would be 0·5 g. per kilo.

Discussion of Results.

The cases of non-diabetic acidosis show much the same range of β-ratio (from 31·6 to 83·8) as do the cases of diabetes (from 40·2 to 83·6). The value 31·6 indicates about one molecule of β-hydroxybutyric acid to two of aceto-acetic acid, while a ratio of 83·6 corresponds to a proportion of about five molecules of the former to one of the latter acid. These are then the extreme limits of composition of the mixtures observed in this series.

The results given in the tables above show that the higher β-ratios occur in the cases excreting the larger total amounts of acetone bodies, but there is no approach to any close parallelism between the two sets of figures. The data given here are of course much too few for the establishment of any rules, but they indicate that a person excreting daily acetone bodies yielding more than about one and a half grams of acetone produces two or more than two molecules of β-hydroxybutyric acid to one of aceto-acetic acid (Table VII).

TABLE VII.

		β-ratio	Daily output of acetone bodies as grams acetone	Alveolar CO_2 mm. Hg.
Diabetes.	*Case* 3	mean 79·1	mean 26·0	—
,,	,, 5	,, 76·7	,, 21·2	mean 26·4
,,	2	,, 74·7	,, 23·3	—
,,	,, 6	,, 74·4	,, 18·2	—
,,	,, 4	,, 74·1	,, 20·3	,, 37·3
,,	,, 1	,, 68·2	,, 2·4	—
,,	,, 14	,, 59·7	,, 0·74	,, 35·6
,,	,, 15	,, 43·0	,, 0·104	,, 38·7
Results of all estimations on 24 hours urine.				
Diabetic		over 75	,, 20·5	,, 32·4
		75 to 66	,, 17·8	,, 35·6
		below 66	,, 0·42	,, 36·7
Non-diabetic		over 66	,, 2·73	,, 32·9
		below 66	,, 0·78	,, 35·8
All cases		over 66	maximum 34·2 minimum 1·42	— —
		below 66	maximum 1·59 minimum 0·019	— —

A shifting of the equilibrium point in the direction of lessened oxidation along with increase in the total production of acetone bodies is evident in Fig. 3, which shows the rapidly developing acidosis in the cases of carbohydrate starvation, while the reverse change is seen in recovery from attacks of vomiting.

The cases of severe diabetes behave somewhat differently in this respect. The results tabulated under Cases 4 and 6 show that fluctuations of 100 % or more in the amounts of acetone bodies produced leave the β-ratio almost unaffected[1]. In these conditions of persistent acidosis, the two acids seem to be produced in very constant proportions which are unaffected by large variations in their total amounts.

One might suppose that the higher proportion of β-hydroxybutyric acid in the urine in the cases of more severe acidosis was due to the washing-out of acetone from the blood into the expired air by the increased lung-ventilation which occurs in such cases [Beddard, Pembrey, and Spriggs, 1908]. If this were the whole explanation, the β-ratio should show an inverse relationship to the alveolar CO_2 pressure, the former rising as the latter falls. The cases given in the tables show that on the whole the higher β-ratios do accompany the lower CO_2 pressures (Table VII), but in detail the relation between the two quantities is very irregular, and there are some quite definite exceptions. Thus Case 4 (diabetes) showed an alveolar CO_2 pressure within or even above normal limits at a time when the β-ratio was above 70, and in Case 19 (starvation) the ratio rose on the third day to about this level, while the alveolar CO_2 was but slightly lowered. Changes in lung-ventilation do not then provide a sufficient explanation of the variations in the proportions of acetone bodies in the urine.

The rise in the β-ratio with increasing acidosis can hardly be due to a shortage of oxygen. If one atom of oxygen were required to convert one molecule of β-hydroxybutyric acid into aceto-acetic acid, the amount of oxygen required to lower the β-ratio for the fifth day in Case 20 (Table V) from the actual value of 75 to 33, would be about 280 c.c., an amount which would be absorbed in less than a minute. Moreover the production of the acid itself increases the supply of oxygen to the lung, by action on the respiratory centre.

The increase in the proportion of β-hydroxybutyric acid when the total amount of the two acids rises suggests that it is the hydroxy-acid which is

[1] Magnus-Levy [1908] has drawn attention to the large variations in the output of acetone bodies which occur even when all the conditions of diet and treatment are kept as constant as possible

the primary product; the evidence upon this point obtained in other ways is not sufficient to decide this question [Dakin, 1912].

The expenses of this work were defrayed by a grant from the Science Committee of the British Medical Association.

Summary.

Estimations are given of the relative amounts of β-hydroxybutyric acid and aceto-acetic acid in the urine in conditions of acidosis. The cases of diabetes show the constancy in the proportions of the two acids to which Neubauer drew attention. In carbohydrate starvation the proportion of β-hydroxybutyric acid rises with the increasing formation of acetone bodies. The estimations on cases excreting more than about two and a half grams of the two acids daily indicate the production of one molecule of aceto-acetic acid to from two to five molecules of β-hydroxybutyric acid.

REFERENCES.

Beddard, Pembrey and Spriggs (1908), *J. Physiol.*, **37**, *Proc.* xxxix.

Dakin (1912), Oxidations and Reductions in the Animal Body. London, Longmans Green and Co.

Graham and Poulton (1913), *Quart. J. Med.*, **7**, 13.

— (1914, 1), *Proc. Roy. Soc.* 87, B, 205.

— (1914, 2), *J. Physiol.*, **48**.

Kennaway (1914). *Biochem. J.*, **8**, 230.

Magnus-Levy (1908). *Ergebnisse der inneren Medizin und Kinderheilkunde*, **1**, 352.

Neubauer (1910), *Verhand. deutsch. Kongr. innere Medizin*, **27**, 566.

Scott-Wilson (1911), *J. Physiol.*, **42**, 444.

Shaffer and Marriot (1913), *J. Biol. Chem.*, **16**, 265.

XLII. SOME NEW PHYSIOLOGICALLY ACTIVE DERIVATIVES OF CHOLINE.

By ARTHUR JAMES EWINS.

(From the Wellcome Physiological Research Laboratories, Herne Hill, S.E.)

(Received July 10th, 1914.)

A short time ago I described [Ewins, 1914, 1] the isolation from extract of ergot, of a new active principle which was identified as acetyl-choline. The action of this base was shown by Dale [1914] to be closely related to that of muscarine, the active principle of the fungus *Amanita muscaria*, to which Harnack [1875] assigned the formula $C_5H_{15}O_3N$, and suggested that it was an oxidation product of choline. "Synthetic" muscarine (pseudo-muscarine), originally obtained by Schmiedeberg and Harnack [1877], and considered by them to be identical with muscarine, but shown by later observers to differ from natural muscarine in physiological action, I have recently shown to be the choline ester of nitrous acid [Ewins, 1914, 2]. The constitution of the natural muscarine, however, still remained (and remains) unsolved, but from the fact that acetyl-choline and pseudo-muscarine, both of which are choline esters, very closely resemble the natural base in physiological action, we were led to suppose that muscarine might also be an ester of choline. Some support was given to this idea when we found that the nitric acid ester of choline, originally prepared by Schmidt and Wagner [1904], still more closely resembled muscarine in its action. It was found, however, that the action of extracts of *Amanita muscaria* was not appreciably lessened by boiling with dilute acid or alkali, so that it seems highly improbable that the natural base is a choline ester.

Since the amount of *Amanita muscaria*[1] at our disposal was too small to admit of isolation of the natural base in a state of purity, or in any quantity,

[1] For an extract of *Amanita muscaria* we are indebted to Dr O. Rosenheim, and for a quantity of dried material to Prof. W. Wiechowski, to both of whom we wish to render our best thanks.

I prepared a number of the simpler choline derivatives which might possibly give a clue to the constitution of the natural base. So far the investigation has not led to the identification of muscarine itself, but certain of the derivatives prepared were of some considerable interest, the ethyl ether of choline $OH . N(CH_3)_3 . CH_2 . CH_2 . O . C_2H_5$ in particular being very closely related indeed in its action to that of the natural muscarine. The bases were in all cases physiologically examined by Dr H. H. Dale, whose results will be published shortly.

The following is a table of the substances examined:

(1)	Acetyl-choline	$OH . N(CH_3)_3CH_2CH_2O . OCCH_3$.
(2)	*Formyl-choline*	$OH . N(CH_3)_3CH_2CH_2O . OCH$.
(3)	Lactyl-choline	$OH . N(CH_3)_3CH_2CH_2O . CHCH_3$. / $OH . N(CH_3)_3CH_2CH_2O . CO$.
(4)	Choline nitrous acid ester	$OH . N(CH_3)_3CH_2CH_2O . NO$.
(5)	Choline nitric acid ester	$OH . N(CH_3)_3CH_2CH_2O . NO_2$.
(6)	Choline methyl ether ..	$OH . N(CH_3)_3CH_2CH_2O . CH_3$.
(7)	Choline ethyl ether ..	$OH . N(CH_3)_3CH_2CH_2O . C_2H_5$.
(8)	*Choline propyl ether* ..	$OH . N(CH_3)_3CH_2CH_2O . C_3H_7$.
(9)	*Choline ether*	$OH . N(CH_3)_3CH_2CH_2O . CH_2CH_2N(CH_3)_3OH$.
(10)	Trimethyl-β-bromo-ethyl-ammonium bromide	$BrN(CH_3)_3CH_2CH_2Br$.
(11)	Trimethyl-β-chloro-ethyl-ammonium chloride	$ClN(CH_3)_3CH_2CH_2Cl$.
(12)	*Trimethyl-β-cyano-ethyl-ammonium hydroxide*	$OH . N(CH_3)_3CH_2CH_2CN$.
(13)	*Trimethyl-β-amino-ethyl-ammonium hydroxide*	$OH . N(CH_3)_3CH_2CH_2NH_2$.
(14)	*Dimethyl-oxazolium hydroxide*	$(OH)\ N(CH_3)_2{-}CH_2$ / $CH_2 . CH_2 . O$
(15)	*Formocholine methyl ether*	$OH . N(CH_3)_3CH_2OCH_3$.
(16)	*Formocholine ethyl ether*	$OH . N(CH_3)_3CH_2OC_2H_5$.
(17)	*Formocholine propyl ether*	$OH . N(CH_3)_3CH_2OC_3H_7$.
(18)	*Formocholine butyl ether*	$OH . N(CH_3)_3CH_2OC_4H_9$.

All of these bases showed considerable physiological activity, those approaching most nearly to muscarine being choline ethyl ether, choline nitric acid ester, and trimethyl-β-amino-ethyl ammonium hydroxide. The curare-like action of all of these is, however, more pronounced than in the case of natural muscarine.

The ethers of formocholine were found to possess an action closely resembling that of the corresponding choline ethers, but were quantitatively much less powerful. Formocholine propyl ether was found to be the most active of the series whereas the isomeric choline ethyl ether was the most active of the choline ethers.

The compounds printed in italics in the above table are new substances. Their preparation and properties are described below. The remaining bases were obtained by the methods previously employed by other workers, notably Schmidt and Wagner [1904] and Nothnagel [1894].

Experimental.

(*a*) *Choline derivatives.*

Formyl-choline, $OH . N(CH_3)_3CH_2CH_2O . OCH$. The base was obtained by boiling a solution of choline chloride in formic acid (D 1·22) under reflux for three hours. The excess of formic acid was removed by distillation *in vacuo*. The residue was dissolved in absolute alcohol and converted into the platinichloride by precipitation with an alcoholic solution of platinic chloride. The precipitate was filtered off and crystallised from hot water in which it is readily soluble. On standing, the crystalline *platinichloride* was obtained in orange-red octahedra. M.p. 255–256°.

0·1111 ; 0·0840 CO_2 ; 0·0450 H_2O. C = 20·6 ; H = 4·5.
0·1087 ; 0·0313 Pt. Pt = 28·8.
Calculated for $(N(CH_3)_3CH_2CH_2O . OCH)_2PtCl_6$. C = 21·4 ; H = 4·2 ; Pt = 29·0.

The *aurichloride* crystallised from hot water in thin golden yellow hexagonal plates. M.p. 175°.

0·1168 ; 0·0488 Au ; Au = 41·8.
Calculated for $N(CH_3)_3CH_2CH_2O . OCH . AuCl_4$; Au = 41·8.

Choline propyl ether, $OH . N(CH_3)_3CH_2CH_2O . C_3H_7$. This was obtained by acting on β-iodo-ethyl propyl ether [Karvonen, 1909] with alcoholic trimethylamine solution at the ordinary temperature. The crystalline iodide of choline propyl ether separated on standing, and was filtered off, converted into the chloride in the usual manner and then into the *platinichloride,* which was obtained crystalline from hot aqueous solution.

Orange red prisms. M.p. 246°.

0·1205 ; 0·1197 CO_2 ; 0·0626 H_2O ; C = 27·1 ; H = 5·75.
0·0922 ; 0·0262 Pt ; Pt = 28·4.
Calculated for $(C_5H_{20}ON)_2Pt\,Cl_6$. C = 27·4 ; H = 5·7 ; Pt = 27·9.

Choline ether, $OH . N(CH_3)_3CH_2CH_2O . CH_2CH_2(CH_3)_3N . OH$. ββ-Diiodo-ethyl ether [Sand, 1901] was treated with a slight excess of alcoholic trimethylamine solution at the ordinary temperature. On standing, the crystalline choline ether iodide rapidly separated, and was filtered off after about twenty-four hours.

The *iodide* crystallises from 95 per cent. alcohol in thin rectangular plates melting at 275°. The salt is very readily soluble in water, but sparingly soluble in absolute alcohol.

0·0736 ; 0·0781 Ag I ; I = 57·3.
Calculated for $C_{10}H_{26}ON_2I_2$; I = 57·2.

The *chloride* forms rhombic plates melting above 280°.

The *platinichloride* crystallises from water in clusters of small prisms melting at 226° (with decomposition). It is sparingly soluble in cold water and almost insoluble in alcohol.

0·1680 ; 0·0534 Pt ; Pt = 31·8.
Calculated for $(C_{10}H_{25}ON_2)$ Pt Cl_6 ; Pt = 32·6.

The *aurichloride* is very sparingly soluble in hot water. On cooling it separates as golden yellow rhombic plates melting at 269°.

0·1300 ; 0·0591 Au ; Au = 45·46.
Calculated for $C_{10}H_{26}ON_2 . 2AuCl_4$; Au = 45·34.

Trimethyl-β-cyano-ethyl-ammonium hydroxide, $OH . N(CH_3)_3CH_2CH_2CN$. β-Chloropropionitrile, $ClCH_2CH_2CN$ [Henry, 1898] was treated with an alcoholic solution of trimethylamine. On standing at the ordinary temperature, the crystalline chloride of the base separated.

The *chloride* was recrystallised from hot absolute alcohol in which it is fairly soluble, whilst it is very little soluble in cold alcohol. The salt separates as prisms melting at 228–229° (with decomposition) and is hygroscopic.

0·1329 ; 0·1272 Ag Cl ; Cl = 23·7.
0·1329 ; 20·8 c.c. N_2 at 19° and 765 mm. ; N = 18·2.
Calculated for $C_6H_{13}N_2Cl$; Cl = 23·9 ; N = 18·8.

The *platinichloride* crystallises from hot water, in which it is moderately easily soluble, in brownish yellow aggregated octahedra, melting at 249°–250° with decomposition.

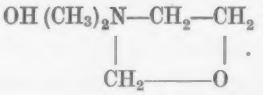
0·1728 ; 0·0542 Pt ; Pt = 31·3.
Calculated for $(C_6H_{13}N_2)_2PtCl_6$; Pt = 30·8.

The *aurichloride* separates from a hot aqueous solution as thin golden yellow needles melting at 213–214°.

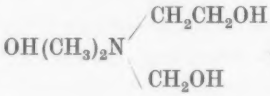
0·1376 ; 0·0601 Au ; Au = 43·7.
Calculated for $C_6H_{13}N_2 . AuCl_4$; Au = 43·6.

Trimethyl-β-amino-ethyl-ammonium hydroxide, $OH . N(CH_3)_3CH_2CH_2NH_2$. This compound was obtained by heating choline nitric acid ester with an alcoholic solution of the calculated amount of ammonia, for several hours at 100°. The solution was then evaporated to dryness, dissolved in a little water and precipitated with a slight excess of an aqueous solution of gold chloride. The precipitated *aurichloride* was filtered off and crystallised from hot, very dilute, hydrochloric acid solution. On cooling, the salt separated as yellowish brown rectangular prisms melting at 263°, very little soluble in cold, moderately soluble in hot water.

0·0999 ; 0·0507 Au ; Au = 50·7.
Calculated for $C_5H_{15}N_2 . 2AuCl_4$; Au = 50·5.

N-Dimethyloxazolinium hydroxide,  . This

substance was obtained in an attempt to prepare an oxy-choline, the constitution of which would be denoted by the formula

 .

On analysis, however, the compound obtained was found to contain one molecule of H_2O less than required by the above formula ; hence it is concluded that ring formation has occurred. This is supported by the fact that heating the chloride of the base with acetyl chloride at 100° for 3–4 hours leaves the compound unchanged. Under similar conditions choline is readily acetylated. The compound was obtained as follows. Dimethylaminomethyl alcohol was prepared by the method due to Henry [1898] and was then treated with one molecular proportion of ethylene chlorohydrin. A vigorous reaction ensued, so that the reaction mixture had to be cooled from time to time during addition of the chlorohydrin. The product was allowed to stand for some time, and then dissolved in a little absolute alcohol. The solution was precipitated by alcoholic mercuric chloride, the very voluminous precipitate filtered off, and crystallised from a large volume of hot water. The crystalline mercury compound was then suspended in water and decomposed by sulphuretted hydrogen. The filtrate was evaporated to dryness, and from the residue, which showed indications of crystallising, the following salts were obtained. The *platinichloride* was readily crystallised from hot water, in which it is moderately soluble (but much less soluble than the corresponding salt of choline). It was obtained as orange red rectangular prisms, melting at 237–238°.

0·1750 ; 0·1257 CO_2 ; 0·0662 H_2O ; C = 19·6 ; H = 4·2.
0·1151 ; 0·0817 CO_2 ; 0·0385 H_2O ; C = 19·36 ; H = 3·7.
0·2134 ; 0·0685 Pt ; Pt = 32·1.
Calculated for $(C_5H_{12}ON)_2\,PtCl_6$; C = 19·6 ; H = 3·9 ; Pt = 31·8.

The *aurichloride* is sparingly soluble in cold, moderately soluble in hot water. It forms golden yellow prisms melting at 279°.

0·1267 ; 0·0567 Au ; Au = 44·8.
Calculated for $C_5H_{12}ON\,AuCl_4$; Au = 44·7.

The *mercuric chloride compound* crystallises from water in hexagonal prisms melting at 244–245°. It appears to have the formula $C_5H_{12}ON\,.\,2HgCl_2$.

(*b*) *Formo-choline derivatives.*

Formo-choline itself was obtained by Schmidt and Litterscheid [1904] who were, however, unable to obtain either acetyl, benzoyl, or lactyl derivatives. Attempts by the writer to prepare the nitric ester of the base by methods similar to those employed for the preparation of the corresponding choline ester proved unsuccessful. It was found, however, that the ethers of formo-choline could readily be obtained by the action of trimethylamine on the corresponding iodomethyl ethers.

Formo-choline methyl ether, $OH . N(CH_3)_3CH_2O . CH_3$. Iodomethyl ethyl ether $I . CH_2O . CH_3$ was prepared according to the method employed by Henry [1893]. For purification it was washed with a very little ice water, dissolved in ether and dried over $CaCl_2$. The ether was removed and the residue distilled *in vacuo*. It boiled constantly at 39° under 20 mm. pressure.

To the ethereal solution of the iodo-ether was added alcoholic trimethylamine, the mixture being kept cool by immersion in ice. A crystalline product consisting of a mixture of formo-choline methyl ether iodide, and trimethylamine iodide, separated immediately. The mixture was suspended in cold absolute alcohol. The bulk of the trimethylamine iodide remained undissolved and was filtered off. The filtrate was evaporated to dryness, and the residue crystallised from a little alcohol by addition of ether. Formo-choline ether iodide was thus obtained as white glistening hygroscopic plates, melting at 84°.

0·1421 ; 0·1428 Ag I ; I = 54·5.
Calculated for $CH_3OCH_2N(CH_3)_3I$; I = 54·98.

The *platinichloride* crystallised from water in columnar aggregates of orange-red rhombic prisms, melting with decomposition at 234°. The salt was anhydrous and sparingly soluble in cold water, but readily soluble in hot water.

0·1028 ; 0·0322 Pt ; Pt = 31·3.
Calculated for $(C_5H_{14}ON)_2PtCl_6$; Pt = 31·6.

The *aurichloride* is moderately soluble in hot water, sparingly in cold. It crystallises in golden yellow needles melting at 135–136°.

0·1212 ; 0·0536 Au ; Au = 44·2.
Calculated for $C_5H_{14}ON\ AuCl_4$; Au = 44·4.

Formo-choline ethyl ether, $OH . N(CH_3)_3CH_2O . C_2H_5$. Iodo-methyl ethyl ether, $I . CH_2O . C_2H_5$, was prepared by a method analogous to that employed by Henry to obtain the corresponding methyl ether. On distillation *in vacuo*

a liquid was obtained which boiled constantly at a temperature of 44° at 30 mm. The product, however, on analysis was found to be still somewhat impure, but owing to the highly reactive nature of the liquid it was not further purified, but treated at once with trimethylamine, and the product worked up in the same manner as described for the corresponding methyl ether.

Formo-choline ethyl ether iodide was thus obtained as thin lustrous deliquescent plates, melting at 94°.

0·1100 ; 0·1066 AgI ; I = 52·3.
Calculated for $C_2H_5OCH_2N(CH_3)_3I$: I = 51·8.

The *platinichloride* crystallised from hot water in large regular crystals orange-red in colour and melting at 241–242°. It is moderately soluble even in cold water, but quite insoluble in alcohol.

0·1052 ; 0·0321 Pt ; Pt = 30·5.
Calculated for $(C_6H_{16}ON)_2PtCl_6$; Pt = 30·3.

The *aurichloride* crystallised from water in golden yellow leaflets melting at 138–139°.

0·1426 ; 0·0618 Au ; Au = 43·3.
Calculated for $C_6H_{16}ONAuCl_4$; Au = 43·1.

Formo-choline propyl ether, $OH . N(CH_3)_3CH_2O . C_3H_7$. *Iodomethyl propyl ether* $I . CH_2O . C_3H_7$ (b.p. 61–62° at 28 mm.) was obtained by the method employed in the previous preparations, treated with trimethylamine, and the product worked up as before.

Formo-choline propyl ether iodide was thus obtained crystalline from alcohol by addition of ether as long fine needles melting at 108°. The salt was not very deliquescent.

0·1201 ; 0·1112 AgI ; I = 50·0.
Calculated for $C_3H_7OCH_2N(CH_3)_3I$; I = 49·0.

The *platinichloride* was obtained crystalline from hot water, in which the salt is very easily soluble. It is insoluble in alcohol and very little soluble in cold water ; long thin orange-red needles melting at 236–237°.

0·1165 ; 0·0342 Pt ; Pt = 29·35.
Calculated for $(C_7H_{18}ON)_2PtCl_6$; Pt = 29·0.

The *aurichloride* is only moderately soluble even in hot water. It crystallises in golden yellow leaflets melting at 114°.

0·1094 ; 0·0460 Au ; Au = 42·0.
Calculated for $C_7H_{18}ONAuCl_4$; Au = 41·6.

Formo-choline butyl ether, $OH . N(CH_3)_3CH_2O . C_4H_9$, was prepared by the action of trimethylamine on iodomethyl butyl ether $I . CH_2O . C_4H_9$ (b.p.

78–80° at 20 mm.). The *iodide* crystallised in glistening colourless plates melting at 98°.

0·1812 ; 0·1610 AgI ; I = 48·0.
Calculated for $C_8H_{20}ONI$; I = 46·5.

The *platinichloride* crystallises from hot water as orange-red octahedra melting at 243–244°. It is insoluble in alcohol, sparingly soluble in cold, but fairly readily soluble in hot water.

0·0992 ; 0·0276 Pt ; Pt = 27·7.
Calculated for $(C_8H_{20}ON)_2PtCl_6$; Pt = 27·8.

The *aurichloride* is little soluble in cold water, moderately so in hot. It crystallises in golden yellow leaflets melting at 81°.

0·1110 ; 0·0450 Au ; Au = 40·5.
Calculated for $C_8H_{20}ONAuCl_4$; Au = 40·6.

REFERENCES.

Dale (1914), *J. Physiol.* **48**, *Proc.* iii.
Ewins (1914, 1), *Biochem. J.* **8**, 44.
— (1914, 2), *Biochem. J.* **8**, 209.
Harnack (1875), *Archiv exp. Path. Pharm.* **4**, 168.
Henry (1893), *Ber.* **26**, *Ref.* 934.
— (1898), *Bull. Acad. Roy. Belg.* [3], **35**, 360.
Karvonen (1909), *Ber.* **42**, 687.
Nothnagel (1894), *Arch. Pharm.* **232**, 268.
Sand (1901), *Ber.* **34**, 2906.
Schmidt and Wagner (1904), *Annalen*, **337**, 37.
Schmidt and Litterscheid (1904), *Annalen*, **337**, 37.
Schmiedeberg and Harnack (1877), *Arch. exp. Path. Pharm.* **6**, 101.

XLIII. ON THE RESPIRATORY EXCHANGE IN FRESH WATER FISH.—Part I: ON BROWN TROUT.

By JOHN ADDYMAN GARDNER and CONSTANCE LEETHAM.

(*From the Physiological Laboratory, University of London, S. Kensington.*)

In the course of an inquiry, commenced some time ago by one of us, on the toxic effects of various impurities in river water on fish life, data were required on the relative amounts of oxygen absorbed by different varieties of fresh water fish, on the least quantities of dissolved oxygen compatible with healthy fish life, and on the influence of temperature on these quantities. On searching through the literature we were unable to find the systematic information required, and though a number of observations had been made with carp, perch, roach, etc., respiratory exchange had been mainly studied on marine animals. We therefore decided to investigate systematically the common British fresh water fish, and in this paper we give an account of some of our experiments with brown trout (*S. Fario*).

The respiration of fishes was first studied by G. Humboldt and Provençal [1807]. The fishes were placed in a flask of water, the gaseous contents of which had been analysed, and then after an interval a sample of the water was examined and the alteration in its gases determined. The quantity of water was measured, and thus it was possible to estimate the amount of gases absorbed and discharged by fish. A similar method has been used by Vernon [1896] for the measurement of the respiratory exchange in marine invertebrates. Vernon was the first we believe to make use of pumps to determine the oxygen, nitrogen and carbon dioxide. Baumert [1855] improved Humboldt and Provençal's method by passing a stream of water through the flask containing the animals; the gases contained in samples of the water entering and leaving the flask were determined. In recent years a somewhat similar method has been made use of by August Pütter [1908, 1909].

The first elaborate apparatus for measuring the respiratory exchange of fish was devised by Jolyet and Regnard [1876], and was based on the principle of the well-known apparatus of Regnault and Reiset for land animals. This apparatus in improved form has also been made use of by J. P. Baunhiol [1905]. The largest and most complicated apparatus is that devised by Zuntz [1901], who also describes an ingenious apparatus for automatically supplying oxygen as it is used up by the fish.

The method we employed does not differ much in principle from those already used by various observers.

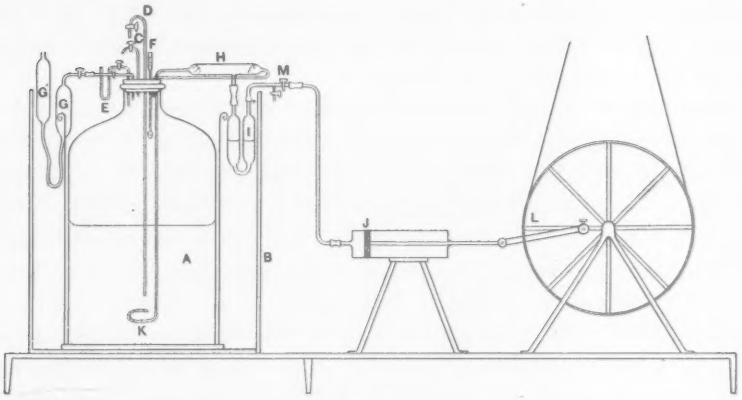


Fig. 1.

The animals were placed in a hermetically closed vessel containing known volumes of water and air, both of which were carefully analysed at the beginning of the experiment. The air was then pumped through the water continuously for a definite period, the oxygen absorbed by the animal being gradually replaced from a reservoir. At the end of the period the air and water were again analysed. The differences in carbon dioxide and oxygen content give the oxygen used by the animal and the carbon dioxide produced in the given time. The apparatus used is simpler than some of the more complex forms described and presents points of novelty.

Apparatus employed.

The apparatus is shown in diagrammatic form in Fig. 1. The receiver *A*, in which the animal was placed, consisted of a large glass bottle of some

50 litres capacity, and with a neck sufficiently large to admit of the animals being easily introduced. This was fixed on a lead base sufficiently heavy to sink it in the water of the thermostat, and provided on the outer edge with stout upright steel rods, which served the double purpose of keeping the bottle firmly in position and affording convenient handles for lifting the apparatus. The bottle was carefully calibrated by weighing it empty and full of water on a delicate machine capable of reading to a gram. The bottle was placed in a thermostat consisting of a large wooden tub *B* containing water. The temperature of the water in the tub was regulated by means of ice or warm water circulating in a coil of pipe, or by an electric lamp—whichever happened to be most convenient for any particular experiment. The water was stirred by bubbling air through from a cylinder. These accessories are not shown in the diagram for the sake of clearness.

The neck of the bottle was closed by a stout rubber bung, through which passed (1) a capillary tube *C*, for withdrawing samples of air, fitted with a three-way tap, so that the capillary could be filled with mercury ; (2) a capillary tube *D* for withdrawing samples of water, also fitted with a tap and reaching to the middle of the water in the bottle ; (3) a delicate manometer *E* which could be closed by a tap, shown diagrammatically ; (4) a thermometer *F*, of the kind used in incubators, for recording the temperature of the air ; (5) a tube leading to the oxygen reservoir *G* ; (6) two tubes communicating with the pumping mechanism *H*, *I* and *J*. One of these tubes connected the end of the valve chamber *H* with the air in the bottle and the other, connected to the opposite end of *H*, reached to the bottom of the water and terminated in a perforated glass spray *K*. The mechanism for pumping the air in the bottle *A* through and through the water consisted of a cylindrical valve chamber *H* of about 80–100 cc. capacity containing two valves opening as indicated. Each valve was made by a disc of thin sheet rubber fitting over a brass nose, and proved very effective. The body of the valve chamber was connected to one limb of a large U-tube of the shape indicated containing mercury, the other limb of which was connected by a brass tube to an accurately made single action brass pump *J*. By means of this pump the mercury was made to rock up and down in the U-tube, and by means of the valves in *H* ultimately sucked air from the upper part of *A* and forced it through the spray *K* in the lower part. The amount of displacement of mercury was regulated by the length of stroke of the pump, which was varied by clamping the end of the connecting rod further from or nearer to the axis of the wheel *L*. The wheel *L* was geared by means of a reducing pulley

(not shown in diagram) to main shafting. In most experiments it was found convenient to arrange so that the piston of the pump gave 25 strokes per minute, each stroke displacing approximately 100 cc. of mercury in the U-tube. The side tube *M*, fitted with a tap, afforded a convenient fine adjustment. The oxygen reservoir *G* consisted of an accurately measured tube containing the amount of oxygen required in any particular experiment. Reservoirs of various sizes were provided. The oxygen can be as a rule most conveniently delivered by hand, by raising the companion tube *G'* containing water and opening the tap, a few cc. at a time at regular intervals, or if desired the tap can be left open and the oxygen delivered continuously at any desired rate by dropping water into *G'* by means of a water clock or other arrangement. Usually, however, it was found quite satisfactory to do this by hand.

The volume of the gas in the valve chamber *H*, with its various tubes, and in the left hand limb of the U-tube *I*, when the mercury was drawn, as described later, to a certain mark *Z* on the narrow upper part of the right hand limb, was accurately determined, and also the volume of the glass of the various tubes dipping into the bottle.

In performing an experiment, the bottle *A* was weighed, and water poured in and its volume determined by weighing again. The bottle was then placed in the thermostat and left for the temperature to adjust itself. The temperature of the water was then read, also that of the thermostat, which should be approximately the same. The fish to be experimented on were then taken from their tank, washed with pure water and weighed. They were then put into the bottle. If the temperature of the water in the tank is much different from that to be used in the experiment, the temperature of the fish must be slowly brought to that required in the experiment while in the pure water serving to wash them. This precaution seems to be very necessary. The stopper was then adjusted as in the diagram, and samples of air and water withdrawn from *C* and *D* by displacement of mercury. The apparatus for collecting the sample of water is given in Fig. 2. It consisted of a cylindrical vessel *a* fitted with a three-way tail tap at *b*, and at the other end with a capillary delivery tube *c*: *a* and its tubes were filled with mercury and connected at *b* with the tube *D* of the fish bottle. By suction through the tail of the tap *b* the capillaries were filled with the water and the taps adjusted. The bottle *d* was then fixed to the delivery tube *c* by a rubber tube and by sucking gently at *e* the mercury flowed from *a* and was displaced by the water from the fish bottle. The sampling tube *a* was

accurately calibrated and thus a known sample of the water was obtained quite free from air bubbles and protected from contact with air. After taking samples, the tap *D* (Fig. 1) was closed, and by suction at the tap *M* by means of a small hand pump (not shown) the mercury in *I* was drawn to the point *Z*. The manometer tap was opened to see that the gas in the bottle was at atmospheric pressure, and the tap of the air tube *C* was closed. The thermometer *F* was then read, and the time taken. The pump *J* was now started, and the air of the bottle *A* sprayed through the water. During the experiment the oxygen used by the fish was renewed from *G* as described, care being taken that the pressure in *A* as recorded on the manometer remained approximately the same. At the end of the experiment the pump was stopped,

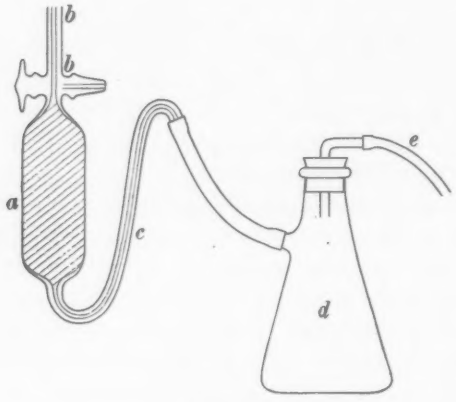


Fig. 2.

and the mercury in *I* sucked again to *Z* and the manometer read. The temperature recorded by *F* was again taken, and samples of air and water withdrawn as before. The time at which the water sample was taken was noted, and the temperature of the water in *A* and in the thermostat read. The gases in the water samples were pumped out by means of the modified form of Toepler pump without taps or joints, devised by Buckmaster and Gardner [1910] for blood-gas work. The samples of water were introduced into the pump through the barometer direct from the sampling tube (Fig. 2) by displacement of mercury in the manner described in the paper referred to. As it was found better to determine the whole of the carbon dioxide, free and combined, in the water samples, 10–15 cc. of 80 % phosphoric acid were

introduced into the pump before the water samples. This pump, with some further modifications introduced by Buckmaster and Gardner [1912], proved very suitable and convenient for this work, as it was not found necessary to cut the apparatus down after each experiment for cleaning purposes as in the case of blood work.

The gas analysis apparatus employed for the analysis of the air samples and the gases pumped from the water was the well-known form of Frankland, as modified by W. A. Bone.

Various difficulties were encountered in perfecting the apparatus, and of these we may mention one, an apparent "diffusion" of nitrogen through the rubber cork and rubber joints of the apparatus. This was more noticeable when there was any difference of nitrogen partial pressure on different sides of the rubber, as for instance in experiments in which oxygen, or mixtures of oxygen and nitrogen richer in oxygen than air, were used in the bottle. This was obviated by covering the upper and lower faces of the bung with tin foil, and by thickly painting the bung and all rubber joints with the acid proof enamel used by electricians for painting the connections of accumulators. We also found it very necessary to take the utmost care in the measurement of the temperature and pressure of the air in the bottle, and to arrange that the temperatures of the inner water and the thermostat were very nearly the same all through. In some of our earlier experiments we modified the chamber *H* by introducing a device by which the carbon dioxide could be absorbed from the air passing through, and thus the accumulation of carbonic acid in the water prevented. This carbon dioxide was afterwards estimated chemically. It was found however that comparatively little carbon dioxide was swept out of the water by the air current, and that we had merely introduced another estimation of somewhat doubtful accuracy with little if any beneficial effect. Further the experiments of Krepzis [1901], and also of Hans Winterstein [1908], have shown that fish such as perch and roach are not apparently affected by far larger quantities of this gas than were ever produced in our experiments. The procedure was therefore discontinued.

An example will make clear the method of calculation:

Weight of water taken 29526 g.; volume of air above the water at the beginning of experiment 17299·7 cc.; temperature 21·2° C., barometer 762·5 mm. The volume of dry air at 0° and 760 mm. was therefore 15710·9 cc. 13 fish were used weighing 300 grams and the experiment was commenced at 11.15 a.m.

A sample of the air taken for analysis measured 20·09 cm. at 20·42° and constant volume; after treatment with potash it measured 20·00 cm. at 20·40°; and after alkaline pyrogallate 15·86 cm. at 20·32°. The total air therefore contained 69·3 cc. of carbon dioxide, 3234·4 cc. of oxygen and 12407·2 cc. of nitrogen at 0° and 760 mm.

53·5 cc. of the water yielded to the pump 8·39 cm. of gas at 20·25° and constant volume (48·65 cc.); after potash the pressure was 1·53 cm. at 20·20° and after alkaline pyrogallate, 1·13 cm. at 20·20°.

The total volume of water (29526 cc.) therefore contained 2257·2 cc. of carbon dioxide (free and combined), 130·4 cc. of oxygen and 371·7 cc. of nitrogen at 0° and 760 mm.

The experiment was continued until 6.15 p.m., and during this time 278·96 cc. of commercial oxygen, measured at 21·2° and barometric height 762·5, were added. 13·97 cm. of this oxygen at 20·3°, after treatment with pyrogallate, measured 0·42 cm. at 20·3°. The dry gas added contained therefore 245·7 cc. and nitrogen 7·6 cc. at 0° and 760 mm. After the end of the experiment the volume of the gas above the water was 17299·7 cc., the temperature was 21·2° and the barometric height 762·5. The manometer showed a positive pressure of + 0·15 cm. The volume of the dry gas at 0° and 760 mm. was therefore 15698·1 cc.

11·9 cm. of the air at 20·88° measured after treatment with potash 11·74 cm. at 20·7°, and after alkaline pyrogallate 9·42 cm. at 20·8°.

The total volume of gas therefore contained 201·6 cc. of CO_2, 3066·5 cc. oxygen and 12429·9 cc. of nitrogen. 53·5 cc. of the water yielded to the pump 9·01 cm. gas at 20·8° and constant volume (48·65 cc.), which after treatment with potash measured 1·38 cm. at 20·7°, and after alkaline pyrogallate 1·09 cm. at 20·55°.

The total water therefore (29526 cc.) contained 2504·5 cc. CO_2, 95 cc. oxygen and 358·1 cc. nitrogen at 0° and 760 mm.

Adding therefore the volumes of carbon dioxide, oxygen and nitrogen together we get:

	At beginning of experiment	At end of experiment	Difference
CO_2 ..	2326·5	2706·1	+379·6 cc.
Oxygen ..	3610·5	3161·5	−449·0 „
Nitrogen ..	12786·5	12788·0	+ 1·5 „

$$\text{Oxygen consumed per kilogram per hour} = \frac{449}{0{\cdot}3 \times 7} = 213{\cdot}8 \text{ cc.}$$

$$\text{Oxygen consumed per fish per hour} = \frac{449}{13 \times 7} = 4{\cdot}63 \text{ cc.}$$

$$\text{Respiratory quotient} = \frac{379{\cdot}6}{449} = 0{\cdot}84.$$

Sources of error. In respiration experiments care should be taken that towards the end of the experiment there should be no oxygen deficiency. It is well-known that fish can stand a moderate diminution of the oxygen content of the water without harm, but it is not advisable to allow the oxygen to diminish during the experiment by more than one-third of its original value, and even this should be avoided if possible. It might be thought that a rapid spraying of the air, in which oxygen was continually renewed, through the water, as in our experiments, would effectively prevent this diminution of the oxygen content of the water. This was not however the case, as diffusion of a gas through water is a slow process. If the oxygen content of a bulk of water such as was used in our experiments is allowed to diminish, it takes a surprisingly long time before equilibrium is again established by even a very vigorous spray. It is important therefore not to place too many fish in a given bulk of water. The experiment quoted in detail above illustrates this difficulty. The number of fish was rather too large, and they used up the oxygen at a somewhat greater rate than could be counteracted by the spraying. Other and more serious sources of error might arise from the consumption of oxygen by organic matter and bacteria in the water, and bacteria introduced into the water by the bodies of fish. The observations of Knauthe [1898] on the oxygen destruction in water in which fish have lived lend strong support to this fear, and any investigation on the oxygen consumption of water animals must reckon with this error and either dispose of it in some way or estimate it quantitatively.

In a botanical metabolic investigation, for instance with germinating seeds, it might be necessary to collect the metabolic products during the course of, say, a week, and from analysis of such products argue as to the changes in the seed. In such a case, obviously, rigidly sterile conditions would be necessary. If, however, in an animal investigation, the necessity for such rigid conditions be postulated, no metabolic researches would be possible, since there are no germ-free animals. Fortunately in our experiments the duration was not so long, and in one, two or three hours and particularly at lower temperatures it did not seem likely that sufficient development of bacteria could take place to introduce any serious error into the results. This conclusion is borne out by the extended series of observations of August Pütter [1909], Zuntz [1901], and others.

To ascertain however, for ourselves, how far serious error due to these causes entered into our results, we made a number of blank experiments under conditions rigidly similar to those in our actual fish experiments, using

both fresh London tap water, and tap water in which trout had been living for from 1·5 to 2·5 hours. The figures are given in the following Table I. As the duration of different experiments varied considerably, the results are reduced to one hour.

TABLE I.

Kind of water	Temperature of water	Duration of expt. in hours	Oxygen lost or gained p. hr. in cc. at 0° and 760	Carbon dioxide lost or gained per hr. in cc. 0° and 760	Nitrogen lost or gained per hr. in cc. at 0° and 760
Tap	10°	3·5	−2·18	+3·94	− 1·03
Tap	15	4·7	−0·25	−7·32	− 6·60
Tap	16	4·5	−2·13	−5·67	− 0·11
Tap	9	2·75	+4·10	+5·21	−33·60
Tap	16·5	3·42	−5·30	+0·63	+13·00
Tap in which fish had lived	15	3·0	−0·07	−6·78	−23·44
,,	10	4·17	−2·08	+1·50	+ 4·80
,,	10	3·70	−3·30	−8·00	− 4·84
,,	10	3·0	+1·97	—	+ 1·97
,,	12	2·32	+3·70	−2·02	− 1·84

The differences should of course be nil, but a glance at the table will show that they are irregularly + and −, so that the source of error in question did not seriously affect our experiments. Nevertheless we adopted all the precautions we could think of to reduce such error. London water, as is well known, is very efficiently filtered and is remarkably free from bacteria, so much so that probably the bulk of those in a sample drawn from the tap are post-filtration organisms. It contains of course a good deal of organic matter in solution, but this appears to be in stable conditions, and on the whole it proved a very suitable medium for the work. In all experiments at summer temperatures we took the precaution to keep the water used in fully oxygenated condition for a day or two in closed vessels before use. The fish were always washed by being allowed to swim about in a considerable bulk of pure water before being placed in the experimental bottle.

The method of pumping out the gases from a small sample of water by means of the blood-gas pump and analysis by the method described proved very satisfactory, and the results of consecutive experiments were sensibly identical. The errors set out in the above table may appear to be very large, but this must of necessity be the case in metabolic experiments of this kind, however accurate the analytical methods adopted. A very minute error in the analysis of the gas from 50–100 cc. of water appears of considerable magnitude when the results are multiplied up to give the gas in 30 litres.

The errors in the oxygen and nitrogen we believe are largely due to the difficulty of determining the exact average temperature of the gas above the water. The carbon dioxide determinations, as will be seen from the table, are less satisfactory than those of oxygen. This is mainly due to the difficulty of estimating very exactly the small quantities of carbon dioxide in the air above the water. Further, in nearly all our experiments the second sample of water was put into the same pump which had been used for the first sample, and which still contained the pumped out water of that sample. The second sample was thus diluted, prior to pumping by its own volume of water, and thus the difficulty of pumping out every trace of carbon dioxide was somewhat increased.

In most of the experiments, as in the one quoted above, when a considerable volume of oxygen was absorbed and carbon dioxide produced, the errors are not serious and make very little difference in reckoning the respiratory quotients. In some of the low temperature experiments, however, when the volume of carbon dioxide produced is small the effect is considerably more marked.

Other chemical methods of estimating carbon dioxide did not seem likely to give as satisfactory results, and indeed are rather put out of court by the fact that brown trout appear to secrete some acid substance which affects the composition of hard water. We have not yet investigated the nature of this substance, nor are we aware of any experiments by other observers. We repeatedly noticed, however, that four or five eight inch trout in some 30–40 litres of water, kept well aerated for a couple of hours, produced enough of this substance markedly to interfere with the action of potassium chromate used as indicator in the estimation of chlorides by silver nitrate, an effect not observed in the case of fish such as dace. The production of this acid appears to be well known to the trout breeders, and Mr F. G. Richmond informs us that our observation fits in with his experience of travelling fish in hard and soft water. The hard water apparently neutralises acids given off by the fish, and this secretion from brown trout is more acid than that from other varieties. We have not yet had an opportunity of experimenting with other varieties than brown trout.

Experimental Results.

Two series of fish were taken for experiment, approximately four inches and eight inches long respectively. The small fish were fairly uniform in weight, 20–24 grams each, but the large fish showed very considerable variations,

70–150 grams. The small fish were kept in a tank in the laboratory, and the temperature of the water was pretty constant—about 14°–15° C. They were fed on a commercial fish food, consisting mainly of ground up fish, and appeared to eat well.

The large fish were kept in a tank outside, and as the experiments were performed during the winter months, the average temperature of the water was lower and varied considerably. They were fed on live minnows, and this was the only food they would take at this period.

The results of our experiments are gathered together in the following Table II. We do not give the full protocol of each experiment, since, as the same apparatus was used all through, the details do not differ very much from those in the example already given in full.

Discussion of Results.

Influence of temperature. The oxygen absorbed appears within limits to be proportional to the temperature, and is approximately doubled for an increase of ten degrees. This is best seen in the case of the small four inch trout, partly because under the conditions of experiment each small animal had a freer range of movement, but mainly because the same animals were used in each experiment and in relatively large numbers, so that the influence of individual idiosyncrasies was to a considerable extent got rid of.

In the case of the larger fish, experiments 5–20, the results are very variable. The eight inch trout differed from one another much more in weight than the four inch, only two or four animals were used in each experiment, and in different experiments different animals were of necessity used. These were taken from the stock in the tank indiscriminately, the only precaution being that only healthy fish were used. Our tank accommodation and water supply did not permit of our keeping more than eight or ten of these fish at a time in healthy condition, in fact it was found better to have a smaller number than this. The fish were therefore frequently renewed from the farm as they died off or were killed for experimental purposes. Some of the fish used were therefore fresh from the farm and had not yet got used to their surroundings, others had been in the tank for a considerable time. This would no doubt have an influence. Most of the experiments were done in the winter months, and the animals were kept in an open tank with running water outside. On the very cold days the fish were sluggish and did not feed much, but even in the warmer periods when they appeared more ravenous they would only take live minnows. In the spring and summer they would

TABLE II.

No. of experiment	Temperature of water °C.	No. of fish	Total weight of fish in grams	Average weight per fish in grams	Oxygen absorbed per kilo per hour in cc. at 0° and 760	Oxygen absorbed per fish per hour in cc. at 0° and 760	Carbon dioxide evolved per kilo per hour in cc. at 0° and 760	Respiratory quotient	Remarks	
1	7·5– 7·8°	12	276·6	23·0	89·04	2·05	49·4	0·55	Experiments performed end of May and beginning of June, and fish kept indoors and fed on commercial fish food.	
2	15·8–16°	11	264·0	24·0	117·40	2·82	129·0	1·09		
3	21·2–21·4°	13	300	23·0	213·80	4·63	180·8	0·84		
4	25 –25·1°	11	240	21·8	258·87	5·64	209·2	0·81		
			Experiments with eight inch brown trout						Large fish in a tank kept outside in running water.	
5	4·1– 4·5°	4	423	106	144·36	15·26	32·18	0·22	Fresh fish.	Dec.
6	6·3– 6·6°	2	235	118	93·21	10·95	11·43	0·12		Dec.
7	5·9– 7·2°	2	235	118	105·26	12·37	1·13	0·01	Same fish as in (6).	Dec.
8	6·3– 6·5°	3	285	95	130·6	12·40	17·79	0·14		Nov.
9	6·3– 6·4°	3	322	107	71·58	7·68	27·78	0·39		Dec.
10	6·1– 7·0°	4	354	88·5	96·44	8·53	93·19	0·96	Fish in tank several days and tank at same temp. as experimental water	Dec.
11	6·3– 7·4°	5	403	80·6	76·20	6·14	74·3	0·98		Jan.
12	10°	3	285	95	230	21·85	172·4	0·75		Nov.
13	12·5–12·6°	3	322	107	149·76	16·07	129·3	0·86		Dec.
14	16 –16·6°	4	325	81	206·45	16·77	131·5	0·64	New fish.	Nov.
15	16·8°	3	267	89	224·78	20·45	213·6	0·95		Nov.
16	16·1–16·6°	4	384	96	188·44	18·09	118·1	0·63	Taken on warm day following a spell of cold in which they had not been feeding.	Jan.
17	20·1–20·4°	3	307	102	189·5	19·39	147·8	0·78		Dec.
18	22 –22·7°	3	267	89	218·1	19·40	103·8	0·47	Same as (15).	Nov.
19	21·4–22·2°	4	410	102	186·06	19·0	160·3	0·87	One fish died during experiment towards end	Nov.
20	21·9–22·3°	4	367	92	223·3	20·5	204·5	0·91		Nov.

readily take other food such as worms, flies, etc., but not in the winter. If we take experiments 5–11, done below 7° C. the results are very variable and show nothing as to the influence of temperature, similarly if we take 14, 15 and 16 at 16°, or 17–20 at 22°. If, however, we take the average value of each series, as shown in the following Table III, it will be seen that the

TABLE III.

Number of experiments	Temp.	Average oxygen consumption cc. per kilo per hour
7	4·1– 7·4°	102·5
3	16·1–16·8°	206·5
4	20·1–22°	204·2

oxygen consumption at 16° is double that at about 6°, i.e. it is doubled for a rise of 10° as in the case of the four inch fish. On the other hand the increase appears to stop at this temperature, and the average at 20–22° is approximately the same. It would appear from this that the organism in the case of the larger fish ceases to react to temperature above the limit of 16 or 17°, but it would seem probable that this is more apparent than real, for as is well known, and as indeed we shall ourselves show in a subsequent paper, fish can live and keep well for long periods at a very much lower level of oxygen partial pressure than that corresponding to full saturation; and further the large fish were somewhat restricted in their movements in the experimental tank compared with the small animals, and were consequently less favourably situated for assisting the pumping of water through the gills by swimming movements. If we make the assumption that a fish absorbs the whole of the oxygen from the water passing through its gills, then calculating on the basis of Winkler's figures [1905] for the oxygen content of water saturated with air at various temperatures, a four inch trout at 7–8° would need to pump 242–248 cc. of water through its gills per hour, or about 4 cc. per minute, while at 21° he would need 743 cc. per hour or 12·4 cc. per minute, and at 25°, 977 cc. per hour or 16 cc. per minute. On the same basis an eight inch trout would pump, at 6°, 1207 cc. per hour or 20 cc. per minute, while at 16° he would require 2676 cc. per hour or 44–45 cc. per minute. The above assumption is perhaps scarcely warrantable, so that these figures must be regarded as minimum values. It is therefore perhaps not surprising that the large fish should apparently cease to react to increases of temperature at a lower temperature than the small fish. On the other hand higher temperatures appear to be dangerous or fatal to trout. On

attempting to determine the oxygen absorption for eight inch trout at 25° C., to compare with result No. 4 for four inch trout, of the three fish used in the experiment one died in about two minutes, though the water was fully oxygenated, and the other two turned over on their backs in about ten minutes, and would no doubt have died, but on adding cold water they recovered completely and rapidly and appeared quite well the next day. This result was not due to any sudden change of temperatures as the fish were carefully warmed up beforehand during the course of one hour. It must be noted, however, that the small fish which stood the 25° temperature during an experiment of several hours duration without apparent harm had been living for some days in a tank in the laboratory in relatively warm water (14–18°), whereas the large trout had been living in a tank outside at ordinary winter temperatures of 9–10° or thereabouts. It is possible that had they been living under summer conditions for a long time they might have stood the high temperature better. In another experiment owing to the accidental use of an imperfect thermometer, four eight inch fish were plunged into well oxygenated water at 33° C. The fish gave a few violent leaps and collapsed, as one might perhaps imagine a warm blooded animal doing on falling into boiling water, and were all quite dead in under one minute. The water afterwards was found to be quite frothy as though it contained some saponaceous material.

Influence of size of animal. The oxygen absorption appears to be approximately proportional to the body weight at a given temperature but we have not sufficient data for small animals to show precisely what the average relation is. As far as the experiments go, the rate of oxygen used per kilo per hour at 7° for four inch and eight inch fish is 1 : 1·13, and at 21–22°, 1 : 0·95; at 16° the ratio is different 1 : 1·75, but probably the average of a larger number of experiments would bring this mean to 1 : 1.

Respiratory quotients. If we except experiments 5–9 done at low temperature, the average value of the respiratory quotient is 0·81. The variations lie between 0·5 and 1·01, but the majority lie round 0·8. This value is of much the same order as that found for carp and tench, and for various kinds of marine fish by Jolyet and Regnard, and others, and the variations fall within similar limits (*vide* table in Schäfer's *Text-book of Physiology*, vol. I, p. 703).

In experiments 5–9 done at low temperatures, and on fish which had been living in a tank outside during a spell of cold weather, the respiratory quotient shows a remarkable drop, and its average value in the five experiments is only 0·18. We do not profess that any of the five individual values

possesses any high degree of accuracy, for the quantities of carbon dioxide produced are very small and therefore the error of experiment would be very large, but we think there is no doubt about the drop in the value of the quotient, if for no other reason than that after the first anomalous result the utmost care was taken with the other experiments. The change appears to take place somewhere about 6–6·5° C., and below this temperature the fish appear to be in a condition akin to that of a hibernating mammal. We noticed that the fish in our tanks during cold weather were very sluggish and as far as we could tell did not eat. They left minnows in the tank severely alone, whereas on somewhat warmer days they took them readily. We do not know whether this is the experience of other observers, or whether fish in a state of nature get into this condition. We have, however, made many inquiries amongst fishermen, river watchers and others interested in fish, but have not been able to obtain any definite evidence on the point.

In the hope of ascertaining whether this phenomenon had anything to do with the nitrogen metabolism, estimations were made of the saline and "albuminoid ammonia" contained in the water before and after fish had been kept in it for definite periods at various temperatures (by the conventional methods of water analysis). The results are given in the following table:

TABLE IV.

Temp. of water	Wt of fish used in g.	Saline ammonia given off per kilo per hour	Albuminoid ammonia per kilo per hour
3–4°	329	0·0038	0·002
4–5°	345	0·0058	0·0007
1·8–2·2°	354	0·0058	0·003
	mean	0·0051	0·0019
6·4–6·8°	341	0·0071	0·0045
6·9–7°	383	0·0087	0·0012
	mean	0·0079	0·0028
13–15°	352	0·0064	0·0049
13·7–13·8°	351	0·0118	0·0042
	mean	0·0091	0·0045
21·6°	366	0·0162	0·0039
20·6–21·2°	346	0·0187	0·0061
	mean	0·0117	0·0050

The figures in the last column do not represent of course the total nitrogen excreted, other than saline nitrogen, but merely that portion of the soluble organic nitrogen evolved as ammonia on boiling with alkaline permanganate under conventional conditions. The figures throw no light on the cause of

the low respiratory quotients at low temperatures, though they generally confirm the conclusion that the metabolism of fish increases with rise of temperature.

Estimations were also made of the saline ammonia, urea and water-alcohol soluble substances containing amino-nitrogen in the whole fish in case of animals kept for about a week at definite temperatures. For this purpose the animal was rapidly cut into lumps which were dropped into hot water, rendered faintly acid with acetic acid (1 cc. 50 % acetic per litre). After heating on a water bath for about half an hour, the lumps were pounded up in a mortar and repeatedly extracted with hot acid water, after each extraction the solid matter being filtered on the pump. The aqueous extracts, usually about a litre in volume, were evaporated to small bulk *in vacuo* at a temperature below 40° and made up to a definite volume. Aliquot portions were taken for ammonia and urea estimations which were made by the urease method as described by Plimmer [1914]. The remainder of the solution was mixed with ten times its volume of absolute alcohol and allowed to stand overnight. The alcoholic liquor was then filtered, the insoluble residue washed with alcohol, and after being rendered alkaline, the alcohol was distilled from the filtrates *in vacuo*. The alkaline aqueous solution so obtained was then made up to a definite volume and analysed for amino-nitrogen by van Slyke's method. The results are given in Table V.

TABLE V.

Temp. average during week	Amino-nitrogen of alcohol soluble substances in g. of N. per 100 g. of fish	Urea-nitrogen in g. per 100 g. of fish	Saline-nitrogen as ammonia in g. per 100 g. of fish
4°– 5° C.	0·082	0·00028	0·0045
4°– 5° C.	0·077	0·0033	0·0194
8°– 9° C.	0·0886	0·0007	0·0139
9°–10° C.	0·081	nil	0·015
10° C.	0·0798	0·0027	0·0226

How far the urea and ammonia figures have any value we cannot say, owing to the risk of hydrolysis during the prolonged vacuum distillations.

The amino-nitrogen figures are very constant at the various temperatures, and the ammonia figures fairly so. We think it is evident from these experiments that low respiratory quotients have no direct connection with the nitrogen metabolism or the quantities of soluble nitrogenous substances in the tissues.

A possible explanation may perhaps be found in the fat. If at the low temperatures the animals are in a state of hibernation or starvation, they may be living on their fat and partially converting it into glycogen and sugar. Some such process might result in a low respiratory quotient, as indicated in the following equation :

$$2C_3H_5(C_{18}H_{33}O_2)_3 + 64O_2 = 16C_6H_{12}O_6 + 18CO_2 + 8H_2O$$
$$CO_2/O_2 = 18/64 = 0{\cdot}281.$$

Owing to the fact that we had no spell of frosty weather this year after the end of January we were unable to test the validity of this hypothesis, but we hope to be able to investigate this question more closely during the coming winter.

We take this opportunity of expressing our thanks to the Government Grant Committee of the Royal Society for aid in carrying out this work, and also to Mr F. G. Richmond of the Surrey Trout Farm for help in obtaining supplies of fish and for much valuable advice.

REFERENCES.

Baumert (1855), *Chem. Untersuch. u. d. Respir. d. Schlammpeitzgus.* Breslau, 524.
Baunhiol (1905), *Bull. scient. de la France et de la Belgique*, **39**, 227.
Buckmaster and Gardner (1910), *J. Physiol.* **40**, 374.
—— (1912), *J. Physiol.* **43**, 406.
Humboldt and Provençal (1807), *Mem. de la Soc. de phys. et de Chim. d'Arcueil*, Paris, **2**, 359.
Jolyet and Regnard (1876), *Compt. Rend.* **82**, 1060.
—— (1877), *Arch. physiol. nom. Path.* **4**, 44.
Knauthe (1898), *Pflüger's Archiv*, **73**, 490.
Krepzis (1901), *Zeitsch. Nahr. Genus.* 631.
Plimmer and Skelton (1914), *Biochem. J.* **8**, 70.
Pütter (1908), *Abhandlungen d. kgl. Ges. d. Wiss. in Göttingen, math. phys. Kl. Neue Folge*, **6**, 1.
—— (1909), *Zeitsch. allgem. Physiol.* **9**, 174.
Vernon (1896), *J. Physiol.* **19**, 18.
Winkler (1905), *Landolt, Börnstein, Meyerhoffer, Tabellen.* 3 Auflage, 184.
Winterstein (1908), *Pflüger's Archiv*, **125**, 73.
Zuntz (1901), *Arch. Anat. Physiol.* 543.

XLIV. A METHOD FOR THE ESTIMATION OF SUGAR IN BLOOD WITH OBSERVATIONS ON SOME MODERN METHODS.

By ARTHUR DUNCAN GARDNER and HUGH MACLEAN.

(*From the Chemical Pathology Department, St Thomas's Hospital.*)

(*Received July 14th, 1914.*)

Until recent years, exact determinations of the sugar content of blood have been attended with great difficulty. The high protein content of blood rendered the separation of the sugar a tedious and uncertain process and even when this was accomplished, there was no very accurate method for the quantitative estimation of small amounts of sugar. In order to ensure fair accuracy, large amounts of blood had to be taken, and this fact in itself prevented complete investigation of the blood in many cases. A few years ago Rona and Michaelis [1908] made use of dialysed iron to separate the blood proteins and a combination of this procedure with Bertrand's [1906] method for the estimation of sugar gave excellent results. The disadvantage, however, was that twenty grams or so of blood were required and the method was rendered tedious by the filtering and evaporation necessary; on this account the number of observations that could be carried out in a day was very limited. In many cases in which the estimation was of the greatest importance it was difficult to obtain the necessary amount of blood; this often happened in the case of the human being, and in smaller animals in which the total blood volume is low. In cases where the necessary amount of blood was available the method gave very good results, and although many other methods have been suggested and used from time to time, our experience of them suggests that, on the whole, the combination of dialysed iron and Bertrand's sugar estimation is undoubtedly the best for blood.

Attempts have been made to estimate the sugar in much smaller amounts of blood than the twenty grams or more used in the above method. Recently Michaelis [1914] has published an account of a method by which he claims to

be able to estimate the sugar in 1 cc. of blood. We have tried to use this, but although the directions given were scrupulously followed, the results in our hands were not satisfactory. The principle is the same as that of the older method, but in this case the precipitate of cuprous oxide is separated by the centrifuge. In the case of small amounts of sugar we were often unable to get any precipitate, and sometimes in the case of two parallel experiments carried out in exactly the same way a precipitate would be obtained in one of them while not a trace was evident in the other one. As we were quite unable to overcome these obstacles, we did not persevere with the method, but this process of sugar estimation might be recommended, if some satisfactory means of separating the reduced cuprous oxide could be found, both on account of the rapidity with which results are obtained and the small volume of blood necessary.

Another method based on a different principle is that of Bang [1913, 1914]. This method we have investigated and used with very many specimens of blood in which the total sugar present was estimated by other means. Using a few modifications which will be described later, we obtained very good results and consider this method of Bang a very valuable one where only traces of blood can be obtained. It is obvious however that the small amount of blood used in the estimation (an average of 0·1 gram) tends to exaggerate any error when the total sugar is expressed in percentage; again a special torsion balance is required and in practice the method takes considerable time.

In certain investigations of the blood sugar of patients—both normal and diabetic—which we have been carrying out, it was necessary to procure samples at frequent intervals and to obtain sufficient blood to secure controls. What was required was some method by which the amount of sugar in about 2 cc. of blood could be estimated quickly and accurately.

Various attempts were made to evolve some method or modification of a method whereby this could be done, but at first the results were not very satisfactory. By using a very dilute solution of permanganate in Bertrand's method and working out a special table, fairly accurate results could be obtained with a few cc. of blood by diluting and clearing with dialysed iron, but the process was tedious owing to the time taken for evaporating the comparatively large volume of fluid to a small bulk; here again it sometimes happened that the fluid which appeared quite clear in large volume showed a distinct turbidity and colouration on evaporation. After many experiments we have now evolved a modification by which the percentage of sugar in 2 cc. of blood may be estimated in from 15 to 20 minutes. No special

apparatus is necessary except a filter pump and even this can be dispensed with. The principle of the method is based on the fact that when blood is treated with a small volume of water and haemolysed it forms a thick paste on the addition of dialysed iron and sodium sulphate. This paste will not filter, but the greater part of the liquid can be expressed by placing a piece of strong linen or cotton material in a funnel and putting a filter paper in position above it, pouring the pasty mass on to the filter paper, bringing the cloth over the latter and gradually pressing out the contents, if possible without bursting the filter paper. In this way a great deal of the liquid is procured, and since experiment shows that the concentration of sugar in the expressed fluid is the same as that in the original paste, an estimation of the sugar in an aliquot part of the fluid gives the factor necessary for estimating the percentage in the blood.

In carrying out the method it generally happens that the fluid so expressed is somewhat turbid and unsuitable for direct estimation of its sugar content; in order to obtain a clear fluid it is necessary to press very gently and carefully and even then the result is often unsatisfactory. There is however no necessity to obtain a clear fluid at this stage; no matter how turbid the fluid is, it is only necessary to pass it through a small filter paper in order to get a water-clear liquid. This filtration requires only a minute or two, so that in practice one presses out as much fluid as possible, folding the cloth over the filter paper and wringing it hard with the hands towards the end of the process. The more or less turbid fluid obtained is then filtered. The whole operation does not take more than about six minutes; in fact we have occasionally measured the blood, performed the necessary manipulations and had the liquid ready for direct estimation of the sugar in five minutes. This compares very favourably with the older methods where long periods are required for filtration. Besides, the only apparatus necessary is a pipette for measuring the blood, a funnel, a mortar and pestle and the requisite flasks and filtering materials, and this is important from the clinical point of view.

After devising means by which an aqueous solution ready for the estimation of sugar could be quickly prepared from blood, the next point that arose was the determination of the amount of sugar in the liquid. Bertrand's method was tried and gave good results with high sugar values, but with small amounts of sugar the method as usually carried out with the filter pump was found to be absolutely unreliable. With the traces of sugar present in these experiments (varying from 0·25 mg. to 6 mg.) it was

comparatively easy to obtain accurate results with the higher values, but, in the case of the lower amounts, great difficulties were encountered. When sugar in very low concentration is boiled with alkaline copper sulphate solution, some of the precipitate is present in a more or less colloidal form so that separation of these particles presents a very difficult problem. In these cases the values obtained depended, among other factors, on the particular sample of asbestos used, while a very slight variation in the size of the perforations of the crucibles produced a marked effect. Using two samples of " asbestos for Gooch crucibles " which seemed quite efficient, a difference of over 40 % of the total amount was obtained. Some samples again absolutely failed to retain any of the precipitate even when a thick layer of asbestos was used. Good values were only obtained when the layer of asbestos was of such thickness that filtration was so exceedingly slow as to render it useless for practical purposes.

As a result of many experiments, we have arrived at the conclusion that in the case of very weak sugar solutions, it is quite impossible to obtain reliable results by using asbestos and the suction pump for separating the reduced sub-oxide in the manner suggested by Bertrand. In ordinary circumstances where the concentration of sugar is fairly high no doubt the method works admirably. With a suitable sample of asbestos, however, we found that fairly rapid filtration could be accomplished by simply packing the asbestos in the crucible and pouring on the liquid to be filtered without using the pump. By this means we obtained excellent results but had great difficulty in procuring a sample of asbestos sufficiently fine to retain the precipitate and yet sufficiently porous to let the liquid through fairly quickly. One excellent specimen for this purpose we found in a sample of " asbestos wool specially prepared for filtering[1]." Since a suitable sample of asbestos is so very important we would suggest the use of this particular variety to anyone making use of the method. The time required for filtration is very short—only about 2 minutes—and the same pad can be used over and over again without being removed from the crucible.

When filtration is complete, the cuprous oxide is washed with water, dissolved in Bertrand's ferric sulphate solution and the sugar estimated in the ordinary way, the special table being used. The steps and details of the method are given below. Since the method is capable of being used clinically we have gone into considerable detail in describing the different manipulations. In order to keep the volume of liquid as small as possible

[1] Sold by Baird and Tatlock.

concentrated solutions of copper sulphate and alkali are used. As weak solutions of potassium permanganate do not keep well, an N/5 stock solution is made and this diluted when required with ten times its volume of water to give an N/50 solution.

Details of Method for estimating Sugar in Blood.

Solutions and substances required:

No. 1. Copper sulphate, 20 g.
Distilled water to 100 cc.

No. 2. Rochelle salt, 90 g.
Caustic soda, 66 g.
Distilled water to 300 cc.

No. 3. Ferric sulphate, 25 g.
Concentrated sulphuric acid, 100 g.
Distilled water to 500 cc.

No. 4. N/5 potassium permanganate solution (6·3200 g. per litre) from which N/50 may be prepared for the experiment.

No. 5. Dialysed iron. Liq. ferri dialysatus, B.P. 1885.

No. 6. Saturated aqueous solution of sodium sulphate.

No. 7. Asbestos. "Asbestos wool specially prepared for filtering."

Together with some pieces of linen about 6–7 inches square, which must be free from starch.

I. *Preparation of Blood.*

In carrying out the method, blood is obtained and if not used immediately is prevented from coagulating by the addition of a trace of potassium oxalate. Two cc. of the blood are then carefully measured into a small mortar by means of a graduated pipette and to this 7 cc. of distilled water are added. The mixture is then stirred gently with the pestle, allowed to stand for half a minute or longer, and 14 cc. of the dialysed iron solution added from a burette. The mixture is then stirred up thoroughly with the pestle for about 10 seconds and 1 cc. of a saturated solution of sodium sulphate run in. On again stirring thoroughly with the pestle a thick pasty mass is obtained which on continued stirring becomes much more fluid. It is now ready for separation of the liquid; the total volume of the mass amounts to 24 cc.

II. *Separation of Fluid Part.*

A small filter paper of about 11 to 12 cm. in diameter is placed in a funnel and rests on a piece of cloth fitted into the funnel so that the cloth which is next to the glass is roughly shaped like the filter paper. As much as possible of the thick mixture in the mortar is transferred to the filter paper, the pestle being used to rub off any adhering to the sides. The upper part of the cloth is then brought over the filter and pressure gradually exerted. After a few seconds the pressure is increased till ultimately the cloth is twisted round and sufficient strength exerted to press out as much liquid as possible. In this way, from 16·5 to 18 cc. of a slightly turbid fluid are generally obtained. Exactly 16 cc. of this are refiltered through a small filter into a graduated 25 cc. cylinder. This fluid, which amounts to $\frac{2}{3}$ of the original volume, and contains $\frac{2}{3}$ of the total sugar content of 2 cc. of blood, is now ready for estimation of the sugar.

III. *Estimation of Sugar in 16 cc. Fluid from II.*

(*a*) *Reduction to cuprous oxide.* The 16 cc. fluid are carefully poured into a small Erlenmeyer Jena flask of 100 cc. capacity. Then 1 cc. of No. 1 copper sulphate solution is measured into the cylinder together with 3 cc. of No. 2 alkaline mixture. This is shaken up and added to the contents of the flask. In this way any sugar remaining in the cylinder is washed out. The mixture is then boiled over a strong flame, using an asbestos wire gauze, for exactly 3 minutes. The flame must be of such a strength that it brings the mixture to the boiling point in a period of from 1 min. 10 sec. to 1 min. 20 seconds. The boiling point is taken as that point at which ebullition becomes general throughout the fluid. Isolated bubbles appear in from 5 to 7 seconds before this, but the boiling point as thus defined is very distinct. After 3 minutes the flask is removed and the liquid is poured on to a specially prepared crucible containing asbestos.

(*b*) *Preparation of crucible and filtration.* By far the most suitable arrangement for the filtration is a Murman crucible[1]. This crucible resembles a Gooch, but a narrow tube leads off from the bottom of the apparatus so that the filtered fluid is delivered directly into the flask ; it is virtually a funnel in which is placed a perforated plate. The stem of the crucible is passed through a

[1] Listed by Gallenkamp and Co.

rubber stopper which fits into a small filtering flask of about 150 cc. capacity. The specially prepared asbestos mentioned above is thoroughly washed in water and then rubbed up gently in a mortar. Some of it is then taken and put into the crucible, a glass rod being used to spread it out evenly; if a pump is available it may be fixed in position in the usual way by suction. On adding some water it swells up, and if the layer be not too thick the water soon begins to trickle through. The necessary thickness is obtained when water at room temperature filters through at the rate of about 10 to 20 drops per half minute. When the crucible is prepared, a little water is poured on, the flask containing the cuprous oxide shaken round a few times and the whole of the contents carefully poured while still hot into the crucible. If the asbestos is of the correct thickness the blue copper solution filters quickly, the whole contents of the flask passing through in about 2 minutes. If less than this time is required more asbestos should be added. The asbestos and sides of the crucible are then washed with a few cc. of hot water with which the boiling flask has already been washed. *During all these manipulations the suction pump must on no account be used.* The crucible is now removed, the contents of the filtering vessel thrown away, the vessel thoroughly washed with cold water and the crucible replaced. The reduction flask is then cooled, 5 cc. of Bertrand's ferric sulphate solution added and the flask shaken to dissolve any traces of cuprous oxide adhering to the glass. About 3 cc. of the ferric sulphate solution is poured carefully on to the sides of the crucible and the asbestos; the asbestos is gently pressed with a glass rod and after a few seconds the last trace of iron solution sucked through by the pump. The remainder of the iron solution is then added and the process repeated. The flask is now washed with exactly 10 cc. of distilled water and this is poured on to the asbestos; after careful manipulation with the glass rod the last drop is sucked through. As with the iron sulphate, the washing here can be advantageously carried out in two parts. The solution is now ready for titration with the N/50 potassium permanganate, which is carefully added from a small 10 cc. burette, until a faint but distinct reddish tinge appears. The number of milligrams of glucose which corresponds to the amount of permanganate required is found on reference to the table. The table was compiled using all the above precautions with the special asbestos described. To be quite comparable with the blood solutions, sodium sulphate was added in each case.

Table showing the amount of glucose equivalent to a certain amount of N/50 Potassium Permanganate solution.

Total volume including alkaline copper sulphate was in each case 20 cc. Filtration under exact conditions described. 5 cc. ferric sulphate used for dissolving cuprous oxide; 10 cc. distilled water used for washing asbestos.

N/50 Perm. cc.	Gluc. mg.	N/50 Perm. cc.	Gluc. mg.	N/50 Perm. cc.	Gluc. mg.	N/50 Perm. cc.	Gluc. mg.	N/50 Perm. cc.	Gluc. mg.
0·53	= 0·25	2·59	= 1·40	4·67	= 2·55	6·28	= 3·70	8·00	= 4·85
0·62	= 0·30	2·66	= 1·45	4·73	= 2·60	6·35	= 3·75	8·08	= 4·90
0·71	= 0·35	2·72	= 1·50	4·80	= 2·65	6·42	= 3·80	8·16	= 4·95
0·82	= 0·40	2·82	= 1·55	4·87	= 2·70	6·49	= 3·85	8·24	= 5·00
0·91	= 0·45	2·93	= 1·60	4·94	= 2·75	6·56	= 3·90	8·31	= 5·05
1·00	= 0·50	3·03	= 1·65	5·00	= 2·80	6·63	= 3·95	8·38	= 5·10
1·09	= 0·55	3·13	= 1·70	5·07	= 2·85	6·70	= 4·00	8·44	= 5·15
1·18	= 0·60	3·24	= 1·75	5·14	= 2·90	6·77	= 4·05	8·51	= 5·20
1·27	= 0·65	3·33	= 1·80	5·21	= 2·95	6·85	= 4·10	8·58	= 5·25
1·37	= 0·70	3·43	= 1·85	5·28	= 3·00	6·92	= 4·15	8·65	= 5·30
1·48	= 0·75	3·53	= 1·90	5·35	= 3·05	7·00	= 4·20	8·72	= 5·35
1·58	= 0·80	3·62	= 1·95	5·42	= 3·10	7·07	= 4·25	8·78	= 5·40
1·69	= 0·85	3·72	= 2·00	5·50	= 3·15	7·15	= 4·30	8·85	= 5·45
1·81	= 0·90	3·81	= 2·05	5·56	= 3·20	7·22	= 4·35	8·92	= 5·50
1·92	= 0·95	3·90	= 2·10	5·64	= 3·25	7·30	= 4·40	8·99	= 5·55
2·05	= 1·00	3·99	= 2·15	5·71	= 3·30	7·37	= 4·45	9·05	= 5·60
2·12	= 1·05	4·08	= 2·20	5·78	= 3·35	7·45	= 4·50	9·12	= 5·65
2·18	= 1·10	4·17	= 2·25	5·85	= 3·40	7·53	= 4·55	9·19	= 5·70
2·25	= 1·15	4·26	= 2·30	5·92	= 3·45	7·60	= 4·60	9·26	= 5·75
2·32	= 1·20	4·35	= 2·35	6·00	= 3·50	7·69	= 4·65	9·33	= 5·80
2·39	= 1·25	4·44	= 2·40	6·07	= 3·55	7·76	= 4·70	9·39	= 5·85
2·46	= 1·30	4·53	= 2·45	6·14	= 3·60	7·84	= 4·75	4·96	= 5·90
2·52	= 1·35	4·62	= 2·50	6·21	= 3·65	7·92	= 4·80	9·53	= 5·95
								9·60	= 6·00

Results of Method.

In investigating the accuracy of the method, a known amount of glucose was added to a blood which had stood at laboratory temperature for several days. The whole of the sugar in this blood had disappeared and on the addition of 0·1 gram glucose per 100 cc. of blood, the following results were obtained:

1. 0·101 %
2. 0·099 %
3. 0·094 %
4. 0·099 %
5. 0·105 %
6. 0·110 %

Average 0·101 %.

Some fresh bloods were also tested; three or four specimens of each were examined as parallel experiments and in all cases the results agreed very well

among themselves. The total sugar concentration was usually almost identical with that obtained by Bang's method or by the old dialysed iron method; the figures generally did not differ by more than about 0·01. Parallel experiments carried out on two different specimens of human blood by this method gave the following values:

(*a*) 0·064 %, 0·067 %, 0·060 % = Average 0·063 %.
(*b*) 0·096 %, 0·101 % = Average 0·098 %.

The total sugar content of two other human bloods was found by this method to be:

(*a*) 0·022 % (diabetic),
(*b*) 0·095 % (normal),

while in the same bloods Bang's method gave (*a*) 0·227 %, (*b*) 0·095 %.

The method as described is not sufficiently delicate to estimate the sugar in blood containing only traces of glucose, *i.e.* less than 0·02 %. In the examination of fresh blood such a contingency never arises, in human blood at any rate, as from 0·05 % to 0·15 % seems to be always present. In experiments on glycolysis, however, it is useful to be able to determine very small traces of sugar, and in order to do this, it is only necessary to add a known amount of sugar beforehand to the solution to be tested. This is most conveniently done by having in stock a special No. 1 copper sulphate solution containing in addition exactly 0·1 % of glucose. Such a solution keeps well and by the addition of the usual 1 cc. to the blood extract, 1 mg. of sugar is added to that actually present in the blood. This plan of adding sugar to increase the sensitiveness of the reaction has been often used in estimating traces of sugars in fluids. Among others, Korowsky [1913] adopted it in the case of blood and Flückiger [1885] in estimating the reducing power of urine.

In carrying out the estimation by this means, one proceeds exactly as before to the point at which the 16 cc. of fluid are poured out of the cylinder into the boiling flask. To ensure exactness, however, the 1 cc. of copper solution is added directly to the liquid in the flask, the cylinder being washed out with the 3 cc. of alkaline solution. The flask is then thoroughly shaken to dissolve the precipitate of copper hydroxide formed and the remainder of the process carried out as described.

In estimating the effects of glycolysis in blood it is often advisable to employ the special copper sulphate solution for the blood when a disappearance of much of the sugar is expected. It can also be used for ordinary

blood but this is not necessary. The only calculation required is the deduction of 1 mg. sugar from the amount actually found.

Using these modifications, it is easy to calculate exactly the percentage of sugar in blood when this amounts only to 0·01 % or less. The table given above extends from 0·25 mg. to 6 mg. of glucose, but the higher values are only obtained in bad cases of diabetes. Normal blood with an average of about 0·1 % glucose requires 2–3 cc. of the permanganate solution which is a convenient amount to work with.

Bang's Micro-method.

As already mentioned, we have found this method exceedingly satisfactory. In the main, we have followed Bang's directions but have introduced certain modifications which in our hands have proved of much value. In this method a small amount of blood is soaked up by a small filter paper and the filter paper extracted with hot potassium chloride solution containing a trace of acetic acid. According to Bang, the protein is coagulated by the hot potassium chloride solution and remains in the paper, whilst the sugar diffuses out into the liquid. We found it impossible, however, to prevent some of the protein passing out into the fluid, and since the presence of traces of protein interferes materially with the result, many of our experiments were rendered valueless.

After various attempts, this difficulty was entirely overcome by putting the filter paper containing the blood into a hot oven (90°–100° C.) for from three to five minutes before extraction. This coagulates the protein so that only on very rare occasions did the fluid show turbidity on subsequent extraction with the potassium chloride solution. If any turbidity did occur it was exceedingly slight and usually insufficient to affect the result of the estimation. This apparently trifling modification has made a marked difference in the practical use of the method and we can now be certain that trouble from protein will not be encountered.

Since the protein is so firmly coagulated we have prolonged the time of extraction to 45 minutes, or better, one hour, in order to allow for complete removal of the sugar. The fluid is brought to boiling point at least twice during each period.

Again, the alkaline copper solution is freed as much as possible from dissolved gases by shaking up and exhausting with a suction pump for a minute or two at the commencement of each series of estimations.

At first we had great difficulty in procuring suitable blotting paper, as many varieties contained something which absorbs iodine even after prolonged treatment with water and acetic acid. The amount of this substance varied even in pieces which had been purified in the same way and all taken from the same sheet. The special samples supplied for the purpose, which are specially purified and ready for use, we have, however, found most satisfactory, and all our later experiments have been carried out with these.

For a few experiments the weight of the blood can be ascertained by any ordinary sensitive balance, but when many estimations are undertaken a special torsion balance is essential.

It is desirable to carry out at least four parallel determinations of each sample of blood and if time allows five or six tubes may with advantage be set up. The method is so extremely delicate that safety is only to be found in numbers and little extra time is involved in setting up a few more tubes.

The mean of three or four satisfactory estimations may be taken as the correct figure.

Frequently it is possible to obtain a series of values which vary very little from the mean. As an example we quote the results of such a case where seven parallel determinations were made. The figures obtained were :—

(1)	0·074 % sugar	(5)	0·076 % sugar
(2)	0·077 % ,,	(6)	0·075 % ,,
(3)	0·074 % ,,	(7)	0·077 % ,,
(4)	0·074 % ,,		

Mean 0·0753 %.

In all cases 0·01 % is subtracted from the final result since experience shows that blood contains a trace of something which reduces iodine and in the case of about 0·01 g. of blood the results obtained are invariably too high by almost exactly 0·01 %.

We have carried out a considerable number of experiments in which the same sample of blood was tested simultaneously by this and by another method. For the control either the old dialysed iron process or the new modified method described in this paper was employed.

The comparative values thus obtained agree within fairly narrow limits, the difference between the estimations being generally not more than 0·01 % to 0·015 %.

Diabetic blood.			*Normal Blood.*	
	Bang's method	New modified method	Bang's method	Old method (dialysed iron)
	0·227 %	0·221 %	0·095 %	0·09 %
	0·237 %	0·224 %	0·092 %	0·10 %
	0·220 %		0·099 %	
	0·225 %	Mean 0·222 %		Mean 0·095 %
	0·226 %		Mean 0·098 %	
Mean	0·227 %			

The method can therefore be said to be quite accurate when carefully carried out if the mean of a number of observations is taken.

The figures given below are picked out at random from a series of observations and show the average deviation of the control experiments.

	Blood (*a*) Normal	Blood (*b*) Normal	Blood (*c*) Diabetic	Blood (*d*) Diabetic	Blood (*e*) Normal
	0·0620 %	0·0636 %	0·334 %	0·300 %	0·0820 %
	0·0507 %	0·0610 %	0·334 %	0·360 %	0·0758 %
	0·0610 %	0·0565 %	0·337 %	0·337 %	0·1020 %
	0·0665 %	0·0489 %	0·329 %	0·369 %	0·0821 %
Mean	0·0600 %	0·0575 %	0·333 %	0·332 %	0·0833 %

Bang's method is indispensable for cases in which only very small amounts of blood are obtainable but for routine purposes it is neither so speedy nor so convenient as the new modified method described.

These two methods fulfil all requirements for the estimation of sugar in blood.

Conclusions.

1. A method is described by which the estimation of sugar in a sample of blood can be carried out in about 20 minutes.

The amount of normal blood required is 2 cc., but in the case of diabetic blood less suffices.

To ensure accuracy it is necessary to follow very carefully the directions given.

2. Bang's micro-method gives good results; coagulation of the blood beforehand as described in the text entirely overcomes the difficulties of interference by protein.

It is advisable to carry out at least four parallel determinations for each sample of blood investigated.

REFERENCES.

Bang (1913), *Biochem. Zeitsch.* **49**, 1.
—— (1914), *Biochem, Zeitsch.* **57**, 300.
Bertrand (1906), *Bull. Soc. Chem.* [3], **35**, 1285.
Flückiger (1885), *Zeitsch. physiol. Chem.* **9**, 323.
Korowsky (1913), *Deutsch. med. Woch.* **34**, 1635.
Michaelis (1914), *Biochem. Zeitsch.* **59**, 166.
Rona and Michaelis (1908), *Biochem. Zeitsch.* **7**, 332; **14**, 479.

XLV. THE APPARENT FORMATION OF EU-GLOBULIN FROM PSEUDO-GLOBULIN AND A SUGGESTION AS TO THE RELATIONSHIP BETWEEN THESE TWO PROTEINS IN SERUM.

By HARRIETTE CHICK.

(*From the Lister Institute.*)

(*Received July 15th*, 1914.)

Introduction.

A great difficulty in completely separating water-insoluble protein has been the common experience of all who have attempted to prepare pure salt-free samples of pseudo-globulin. In the course of fractionating horse-serum for viscosity measurements [Chick 1914], I found that, however carefully the euglobulin of the serum had been previously separated, the final product invariably contained more or less water-insoluble protein. Haslam [1913] devoted much attention to this question and came to the conclusion that the presence of the latter was due to incomplete removal of the euglobulin in the original serum. This is to some extent true, but my experience has led me to believe that the bulk of the water-insoluble material cannot be accounted for in this manner, for a protein, having the characteristics of euglobulin, was found to increase progressively in amount as the purification of the pseudo-globulin proceeded. A small amount of water-insoluble protein can usually be detected immediately after the first separation of the pseudo-globulin by salt precipitation and this does appear to consist of euglobulin originally present in the serum. The comparatively large quantity (in one case, Exp. IV, Table III, nearly one-fifth of the total protein) present at the end of a lengthy dialysis appears, on the other hand, to be derived, at least in part, from a transformation of the pseudo-globulin itself. The experiments about to be described show that, under certain circumstances, pseudo-

globulin may undergo change into a protein possessing the properties of euglobulin and suggest that such may very likely be the origin of the euglobulin present in serum.

Origin of the water-insoluble protein found present in pseudo-globulin.

In one set of experiments (Table I) pseudo-globulin was prepared from diluted horse-serum by the following method, rapidly executed. Ammonium sulphate was first added to one-third[1] saturation, the precipitate separated and the filtrate brought to half saturation of ammonium sulphate, this second precipitate being pressed free from mother liquor and afterwards dissolved in a small amount of water. The presence of a small amount of insoluble protein was always apparent on dilution, especially if the solution were adjusted to a definite, slightly acid, reaction.

This insoluble protein we may regard as euglobulin not thrown out from serum by one precipitation in one-third saturated ammonium sulphate for if, in addition to this process, as much as possible of the euglobulin is previously removed by careful acidification of the diluted serum, the water-insoluble protein which can be detected in the precipitated pseudo-globulin is considerably reduced (see Table II).

When pseudo-globulin (horse), after undergoing one or more re-precipitations, is subjected to a lengthy dialysis to remove ammonium sulphate, it is found to contain a comparatively large amount of water-insoluble protein. As a general rule from one-tenth to one-fifth of the total protein is found to be in this condition. This insoluble material exhibits a striking likeness to euglobulin. It is dispersed by small quantities of dilute acid or alkali and when first separated is dissolved by neutral salts, which latter property, as is the case with euglobulin, becomes impaired with increasing age of the preparation.

It has not been possible to determine with certainty the origin of the whole of this "euglobulin." One view is that it is derived from the pseudo-globulin by some process of "denaturation"[2] or degradation taking place

[1] "One-third" and "one-half" saturation indicate the concentrations of ammonium sulphate obtained when a saturated solution of ammonium sulphate is added to twice its volume or an equal volume of water.

[2] "Denaturation" of proteins cannot at present be identified with any chemical changes in the material. The term "denaturated" (German, *denaturiert*) is applied to proteins possessing a certain group of solubility characteristics, including a total insolubility in water at the iso-electric point, in absence of electrolytes. Solution (dispersion) in water occurs in the presence of acid, alkali or electrolytes, and is associated with the acquisition of an electric charge by the protein particles, while the system displays a high degree of viscosity. The best-known example is that

after separation from the serum and the results of Exps. I–IV set forth in Tables I–III may, on the whole, be said to support this view. On the other hand, it may be urged that the results of these experiments do not necessarily prove that dialysis of salt-precipitated pseudo-globulin is accompanied by its "denaturation," but that removal of all salt may be essential to permit complete precipitation of any "denaturated" protein (euglobulin) present from the beginning. If this is the case, the concentration of euglobulin in serum must be far higher (two or three times) than is generally believed and the usual method of separation, by dilution and acidification, very inadequate.

In order to throw light upon this point, some experiments were made in order to ascertain with what degree of accuracy water-insoluble protein can be estimated in presence of salts and other soluble protein. In one experiment euglobulin was mixed with a sample of thoroughly dialysed pseudo-globulin (from which all insoluble protein had been separated) and ammonium sulphate added; the mixture contained 0·462 % euglobulin, 2·02 % pseudo-globulin and 1·2 % ammonium sulphate. The concentration of insoluble protein was then estimated by the dilution and acidification method described in detail below. The value obtained was 0·337 %, or 73 % of that added. The concentrations of protein and salt in the final acidified, and diluted, solution were 0·048 % and 0·024 % respectively.

A second experiment was made with a thoroughly dialysed pseudo-globulin solution containing 4·77 % total protein, of which about one-seventh was in the "denaturated" condition and present as a precipitate. Estimations of the insoluble protein present in 10 cc. of the above solution were made both before and after addition of 5 cc. of strong ammonium sulphate (10·3 %). In the control, dilution 50-fold, the proportion of insoluble protein was found to be 0·70 %, in the presence of ammonium sulphate the estimated concentration was only 0·23 % and 0·26 % when diluted 50-fold and 100-fold respectively. In this experiment the final concentration of protein and ammonium sulphate was 0·10 % and 0·093 % respectively in the 50-fold dilution and half these amounts in the 100-fold dilution. The proportion of the total insoluble protein which could be precipitated under these conditions was 33 % and 37 % in the two cases.

of proteins which have been acted upon by hot water [see Chick and Martin 1910, 1911 and 1912]. The vegetable globulins and the euglobulin naturally occurring in serum may be regarded as "denaturated" proteins [see Michaelis and Rona 1910, 2]; their property of forming electrically-neutral solutions with certain salts is, however, a special characteristic not shared by heat-"denaturated" proteins.

When whole horse-serum is diluted ten times, as is usual when euglobulin is separated from serum, the total concentration of protein is from 0·6 % to 0·8 % and that of sodium chloride from about 0·08 % to 0·07 %. Under these circumstances, it is quite possible for some euglobulin to escape precipitation when the diluted serum is adjusted to the iso-electric point of euglobulin by the addition of acetic acid. On the other hand, it must be remembered that a chloride is a less efficient solvent for euglobulin than a sulphate [see Mellanby 1905]. Further, there is, as a rule, no suggestion of incompleteness about the process. The euglobulin usually settles with comparative ease, leaving a clear liquid on top.

The results of Exp. III, Table II also throw some light upon this question. In this case a solution of pseudo-globulin made immediately after its precipitation from the horse-serum contained 5·2 % ammonium sulphate, 12·86 % total protein, and only a trace of water-insoluble protein, when subjected to analysis by the method described below. Part of the material was kept in the laboratory as a control and part was dialysed, toluene being present in both cases to prevent decomposition. At the end of two months the water-insoluble material in the control was equal to 8·6 % of the total protein. In the portion which had been dialysed for 51 days the proportion estimated was 14·1 %. This experiment shows that water-insoluble protein, if present, can be detected in presence of electrolytes and other soluble protein. Therefore, a large proportion, at least 61 % (8·6 parts out of a total of 14·1) of the euglobulin found present at the close of dialysis must be regarded as formed from the pseudo-globulin by some process of denaturation taking place after separation from the serum. The remaining 39 % may, on the other hand, be regarded as euglobulin which was present from the first, although it could not be separated in presence of salt. But, from the fact that immediately after preparation only a trace of insoluble protein could be detected in the salt-containing pseudo-globulin, it seems probable that part of this may also be derived from the pseudo-globulin, showing that the denaturation of this protein is accelerated by dialysis.

Experimental methods.

In the experiments set forth in Tables I to III pseudo-globulin was prepared in various ways by salt-precipitation from horse-serum, the details of themethod employed being given at the top of each table. Estimations of the denaturated protein were made before, after, and, in some cases

(Experiments I and II, Table I), during the dialysis, by determining the amount of protein which was precipitated by dilution and adjustment to the iso-electric point.

The method of estimation was as follows. A small portion of the material was taken for a preliminary trial, and largely diluted, so that the protein-content did not exceed 0·1 or 0·2 %. If electrolytes are present the dilution should be as large as possible. 10 cc. of this diluted solution were then placed in each of a series of test tubes and various small quantities of N/100 acetic acid added. If any denaturated protein were present an opalescence or slight turbidity was usually apparent on mere dilution and, when the solution was adjusted to the iso-electric point of the protein by addition of the required amount of acid, a precipitate was formed which aggregated and settled. The series of trial test tubes was always allowed to stand several hours and the composition of the material in the tube in which the precipitate was largest and agglutination most perfect was taken as the pattern for the estimation.

5, 10, or 15 cc. (according to the protein-content) were taken for analysis, diluted suitably and addition made of dilute (N/10 or N/100) acetic acid in the amount necessary for complete precipitation of the denaturated protein, as calculated from the results of the preliminary trial. The whole was allowed to stand 24 hours and then centrifuged, the deposit being transferred to a small, weighed, centrifuge tube, spun down again, washed with distilled water slightly acidified and centrifuged a third time. The final deposit was dried in the weighed tube at 105° until constant in weight.

It is not advisable to permit the estimation to extend over more than one day, as, in some cases, the denaturation of the pseudo-globulin seems to be continued at an appreciable rate in the dilute solution and, when several days are spent over the estimation, higher values are frequently obtained.

If the material to be analysed is slightly acid, small quantities of dilute ammonia must be substituted for the dilute acetic acid. In cases where the material has been dialysed for a long time and the reaction is approaching that of the iso-electric point, dilution with distilled water containing about one-thirtieth of its volume of water saturated with carbon dioxide is often found to be a quick and efficient method of precipitating all the denaturated protein.

If electrolytes are present, denaturated protein may be detected by the above method, but precipitation, under certain circumstances, is incomplete (see above p. 405). In any case it is advisable to make the dilution as great as is possible.

The above method has been described in detail, because the estimation of denaturated protein in presence of other, water-soluble, protein is by no means an easy matter even in the absence of electrolytes, the difficulty being to ensure complete precipitation of the former. When this has not been accomplished, faint turbidity can be detected in the supernatant fluid after centrifuging; in a successful estimation the aggregation of the precipitate should be complete and the top liquor clear. Some of the earlier values for concentration of "denaturated" proteins given in Tables I to III were obtained when the method was not perfected and may be a little inaccurate; the later ones were more satisfactory, as may be seen in some cases where the results of duplicate analysis are inserted.

It was possible that this water-insoluble protein, like euglobulin [Freund and Joachim 1902, Gibson 1906, Banzhaf 1911], might be thrown out by addition of sodium chloride to saturation, and this would have proved a convenient method of separation and estimation. It was tried in Exp. II,

Table I, but without success. After diluting the material 10-fold and saturating with sodium chloride the residual protein (calculated on the original, undiluted, material) was 10·78 %, the total being 11·12 %; that is to say only 0·34 % protein had been thrown down. The concentration of "denaturated" protein as separated by the above method was, however, found to be 0·96 %.

Precipitation by saturated sodium chloride is of little value for the accurate separation of proteins, the amount precipitated depending, to a greater degree than is the case with other salts, upon external conditions, such as the total concentration of protein and the reaction of the material. For example, on one occasion, with horse-serum, the precipitate obtained on saturation with sodium chloride was four times greater when the serum was diluted 1 in 10 than when the dilution was only 1 in 2; in the latter case the higher concentration of total protein protected more than three-quarters of the otherwise precipitable protein. Further, if diluted serum is made slightly acid in reaction, the whole of the pseudo-globulin and albumin is precipitated as well as the euglobulin.

Discussion of Results.

The results of the experiments in Tables I–III suggest that the pseudo-globulin undergoes a progressive denaturation after its separation from the serum. For example, in Exp. I, Table I, the pseudo-globulin immediately after precipitation from the serum showed only a small amount of water-insoluble protein capable of separation by dilution and adjustment of the reaction to the iso-electric point. At the end of dialysis for 10 and 18 days, however, 9·1 % and 12·9 % respectively, of the total protein was found to be in the insoluble condition. It is probable that the ammonium sulphate present interfered with a complete separation of any euglobulin present at the beginning of the experiment, but it is unlikely that the difference in amount of the traces of salt present after 10 and 18 days' dialysis respectively could be responsible for the large difference in amount of insoluble protein found on these two occasions.

Experiment IV, Table III is a second instance. Here material dialysed for five days against tap water contained 11·1 % denaturated protein; after a further dialysis against distilled water lasting 29 days this proportion was increased to 18·1 %.

TABLE I.

Spontaneous formation of euglobulin from pseudo-globulin at ordinary temperature; pseudo-globulin prepared by half-saturation of undiluted horse-serum with ammonium sulphate, after separation of euglobulin by one-third saturation with ammonium sulphate.

Exp.	Material	Date of analysis	Length of dialysis, days	Total protein, %	Water-insoluble protein, %	G. water-insoluble protein per 100 g. total protein	Phosphorus-content, g. P per 100 g. protein: in water-soluble fraction	Phosphorus-content, g. P per 100 g. protein: in water-insoluble fraction
I.	Prepared 2. 12. 13; re-precipitated twice; final precipitate dissolved in water. Dialysed in presence of toluene against distilled water, changed daily, from 5. 12. 13 to 23. 12. 13	5. 12. 13			small amount			
		15. 12. 13	10	14·56	1·33	9·11		
		14. 3. 14[1]	18	13·55	1·75	12·9	0·018	0·171
II.	As in Exp. I but not re-precipitated	6. 12. 13	3	11·17	present			
		26. 1. 14[2]	3	11·12	0·964	8·67		
	(*a*) dialysed against dist. water from 13. 2. 14 to 25. 3. 14	3. 4. 14[3]	43	4·77	0·654	13·7		
		9. 7. 14	43	4·77	0·700	14·7		
	(*b*) as above, but dialysed against dist. water saturated with CO_2 from 13. 2. 14 to 25. 3. 14	26. 3. 14	43	5·51	{0·652 / 0·660}[4]	11·9		

[1] Kept in cold room from 23. 12. 13 until 14. 3. 14.
[2] Kept in cold room from 6. 12. 13 until 13. 2. 14; dialysis then recommenced.
[3] Kept in cold room from 25. 3. 14 until 9. 7. 14.
[4] Duplicate analyses.

TABLE II.

Spontaneous formation of euglobulin from pseudo-globulin at ordinary temperature; pseudo-globulin prepared from diluted (1 *in* 20) *horse-serum by half-saturation with ammonium sulphate, after successive removal of euglobulin precipitates obtained* (1) *by acidification of the serum* (1 *in* 10 *dilution*), *and* (2) *by one-third saturation of the serum* (1 *in* 15 *dilution*) *with ammonium sulphate: once re-precipitated and dissolved in a small quantity of water.*

Exp.	Material	Date of analysis	Length of dialysis, days	Concentration of $(NH_4)_2SO_4$, %	Total protein, %	Water-insoluble protein, %	G. water-insoluble protein per 100 g. total protein	Phosphorus-content, g. P per 100 g. protein: in water-soluble fraction	Phosphorus-content, g. P per 100 g. protein: in water-insoluble fraction
III.	Control, immediately after preparation	4. 2. 14	—	5·23	12·86	trace	—	—	—
	(*a*) Control, allowed to remain at laboratory temperature, in presence of toluene	3. 4. 14	—	5·23	2·86	1·107	8·61	—	—
	(*b*) Dialysed against distilled water, changed daily, in presence of toluene from 2. 2. 14 to 25. 3. 14	30. 3. 14	51	—	4·86	{0·688 / 0·681}[1]	14·1	0·012	0·262

[1] Duplicate analyses.

TABLE III.

Spontaneous formation of euglobulin from pseudo-globulin at ordinary temperature; pseudo-globulin prepared from horse-serum (diluted 1 in 4) by precipitation with one-half saturated ammonium sulphate, this first precipitate extracted with saturated brine, brine-extract precipitated by addition of acetic acid to a concentration of 0·25 %[1]; this second precipitate drained, pressed and mixed with washing soda to a concentration of 3 % and dialysed for five days against tap water.

Exp.	Material	Date of analysis	Length of dialysis, days	Total protein, %	Water-insoluble protein, %	G. water-insoluble protein per 100 g. total protein	Phosphorus-content, g. P per 100 g. protein: in water-soluble fraction	Phosphorus-content, g. P per 100 g. protein: in water-insoluble fraction
IV.	Control	3. 3. 14	(5)[2]	11·72	1·301	11·10	—	—
	(*a*) Dialysed further against dist. water, changed daily, in presence of toluene from 6. 3. 14 to 4. 4. 14	4. 4. 14	29 (+5)	5·87	1·055	17·98	0·0062*	0·156
	(*b*) as (*a*) the distilled water being saturated with CO from 6. 3. 14 to 4. 4. 14	4. 4. 14	29 (+5)	6·40	1·161	18·14	0·0066*	0·098

[1] Gibson's [1906] process.
[2] Against tap water.
* Approximate only (less than 1 cc. N/10 NaOH neutralised).

The results of Experiments I to IV also suggest in every case that the degradation of the pseudo-globulin is approaching a limit. For example, in Exp. II, Table I, after three days' dialysis 8·7 % of the protein was found to be water-insoluble. After an interval of more than two months, including a second dialysis lasting 40 days, the proportion had only risen to 13·7 % and, after having been kept in the refrigerator for a further period of over three months, to 14·7 %. A similar result is shown in Exp. I. In many cases, the experiments were continued beyond the date of the last analysis given in the tables, and the denaturation process was found to be practically at an end. The degraded material was separated as completely as possible by a modification of the method described above, and the clear, top liquor adjusted to the iso-electric point of the denaturated protein and kept for a considerable time in the cold room under observation. In the case of Exps. III and IV (*a* and *b*), only a trace of insoluble material was deposited after periods of two months and one month respectively.

Phosphorus-content of this water-insoluble pseudo-globulin.

The resemblance between the denaturated pseudo-globulin obtained in these experiments and euglobulin led me to make estimations of the phosphorus contained respectively in the insoluble and unchanged pseudo-globulin.

Hardy [1905] demonstrated the presence of phosphorus (0·07 to 0·08 %) in euglobulin (ox-serum) and considered it to be one of the characteristics marking off this protein as a chemical entity. The pseudo-globulin (separated from the serum by saturation with magnesium sulphate) he found to contain a trace only (about 0·009 %) of phosphorus. Haslam [1913] also found phosphorus to be a constant constituent of euglobulin (ox-serum), even after repeated purification, and to be absent from the purest samples of pseudo-globulin which he was able to obtain. In consequence, he used the absence of phosphorus as a criterion of the purity of pseudo-globulin.

In the present case phosphorus was determined by Neumann's method, using N/10 alkali and acid for the final titration, the quantity to be estimated being very small. In some cases where the ammonium molybdate precipitate was only just visible and the total amount of standard sodium hydrate neutralised less than 1 cc., the values obtained must be regarded as approximate only; such values are indicated in the tables by an asterisk.

The results of the phosphorus estimations showed the resemblance between

the denaturated pseudo-globulin and euglobulin to be maintained in this respect also. The former was found to remove almost the whole of the small amount of phosphorus contained in the original pseudo-globulin preparation. In Exp. I, Table I, the water-insoluble protein at the close of the experiment contained nearly 10 times, in Exp. III, Table II, more than 20 times, as much phosphorus as the unchanged pseudo-globulin; in both cases that present in the latter was reduced to a mere trace. In Exp. IV the contrast was even greater; the denaturated pseudo-globulin contained 0·16 % phosphorus, while that remaining with the soluble protein was too small in amount to be estimated with any degree of accuracy.

Two different explanations might be advanced to resume these facts. According to the first, the process described in the last paragraph is merely the separation of the last traces of phosphorus-containing euglobulin derived from the original serum and present from the beginning. On this theory the cessation of the gradual precipitation of insoluble protein would naturally coincide with the disappearance of the phosphorus originally present in the solution. On the other hand, we may regard this precipitate containing protein and phosphorus, which gradually makes its appearance, as the product of a gradual "denaturation" of the pseudo-globulin which requires the co-operation of some phosphorus-containing body (probably a serum-lipoid) which is present in small quantity in the preparations employed. When this serum-lipoid is exhausted, the process is at an end.

The second explanation would appear to be the correct one for the following reason. After the removal of phosphorus from the pseudo-globulin solution, by the separation of the insoluble protein precipitate, addition of a weak emulsion of lecithin, in presence of salt, causes additional formation of a protein resembling euglobulin which can be precipitated on subsequent dialysis.

Handovsky and Wagner [1911] showed that, when emulsions of lecithin, and other lipoids extracted from serum, were added to dialysed serum, a precipitation of the protein took place, which was prevented if salts were present. I have been able to confirm these observations. Addition of a dilute lecithin emulsion produces only slight turbidity when added to solutions of pure egg- or serum-albumin, a fact also noticed by Handovsky and Wagner. In the case of a dialysed pseudo-globulin, an immediate precipitation of protein takes place, which is prevented if a small concentration of salt (1 % sodium chloride), acid, or alkali is present.

I have prepared artificial euglobulin from pseudo-globulin in the following

manner. A small amount of a watery emulsion of lecithin [1] was added to the salt-free material obtained at the close of Exps. III and IV, in which the denaturation process had ceased and from which the insoluble protein had been separated. An immediate precipitate occurred, which was prevented by the presence of alkali in minute proportion or of salt (NaCl), to a concentration of about 1 %. On subjecting the solution to dialysis for 14 days precipitation occurred of a protein, containing phosphorus, and with the characteristics of euglobulin.

Suggestions as to the origin of euglobulin in serum.

On consideration of the results of the foregoing experiments it seems not improbable that euglobulin in serum is a complex material, formed from pseudo-globulin by association with some serum-lipoid, to the presence of which it owes its phosphorus-content.

Hardy [1905], on the other hand, has regarded phosphorus as an integral part of the euglobulin molecule. He [1905, p. 331] was unable to remove all the phosphorus from heat-coagulated euglobulin (ox-serum) by treatment with strong acetic acid and subsequent extraction with alcohol and ether. He therefore concluded that it could not be "due to entangled lecithin." Haslam [1913, p. 514] found that extraction of euglobulin (ox-serum) with boiling alcohol removed a yellow, fatty substance which contained phosphorus, the proportion of the latter remaining in the euglobulin being reduced to about one-half the original. It is, however, quite possible that complete extraction with alcohol or ether is rendered very difficult by the state of aggregation of the protein and that if fresh surfaces could in succession be exposed to the action of these solvents a larger proportion of phosphorus could be removed.

In this connection it is worthy of note that the available analyses of euglobulin, some of which are collected in Table IV, show no approach to constancy in the proportion of phosphorus present. Hardy found 0·07 to 0·08 % phosphorus in euglobulin prepared from ox-serum by dilution and acidification; Haslam found 0·108 % and 0·105 % respectively present in two different samples. In three specimens prepared by the same method from three separate samples of horse-serum, I found 0·12 %, 0·032 %, and 0·065 % phosphorus respectively.

[1] For this preparation I am indebted to the kindness of Dr H. Maclean. The lecithin was prepared by his own method [1914] from heart muscle.

TABLE IV.

Phosphorus-content of euglobulin.

No.	Description of material and mode of preparation from serum	Method	G. phosphorus per 100 grms. protein	mean	Authority
1.	Euglobulin (ox-serum) by dilution and acidification	Neumann	0·11 0·11	0·11	Haslam
2.	Euglobulin (ox-serum) by dilution and acidification	Carius	0·07 0·08	0·075	Hardy
3.	Euglobulin (horse-serum *A*) by dilution and acidification once re-precipitated and washed	Neumann	0·122 0·117	0·119	Chick
4.	Euglobulin (horse-serum *A*) after three subsequent re-precipitations	Neumann	0·092 0·096	0·094	Chick
5.	Euglobulin (horse-serum *A*) as (4), but after extraction of the euglobulin suspension with acetone followed by dry ether	Neumann	0·019* 0·020	0·020	Chick
6.	Euglobulin (horse-serum *B*) by dilution and acidification, twice re-precipitated	Neumann	0·033 0·032	0·032	Chick
7.	Euglobulin (horse-serum *C*) by dilution and acidification. four times reprecipitated	Neumann	0·069 0·062	0·065	Chick

* Less than 1 cc. of N/10 NaOH neutralised.

TABLE V.

Proportion of phosphorus contained in a sample of whole horse-serum and in the various proteins after separation by different methods.

No.	Material	G. P. per 100 g. protein	Mean
1.	Horse-serum: whole serum dried in vacuo at 37°	0·098 0·103	0·100
2.	Total proteins of horse-serum, precipitated by dropping into a large volume of absolute alcohol; washed with alcohol and ether; and dried at 105°	0·027 0·018*	0·027
3.	Total proteins coagulated in hot water, coagulum washed with water, alcohol and ether; dried at 105°	0·004* 0·008*	0·006*
4.	Euglobulin prepared from No. 6 by one-third saturation with ammonium sulphate (re-precipitated six times)	0·071	0·071
5.	Euglobulin prepared by dilution and acidification of the original serum (re-precipitated four times and washed twice with distilled water)	0·069 0·062	0·065
6.	Total globulin, prepared by one-half saturation with ammonium sulphate (re-precipitated six times)	0·023	0·023
7.	Serum albumin precipitated in the filtrate from No. 6 by saturation with ammonium sulphate (re-precipitated five times)	0·032	0·032

* Approximate only, less than 1 cc. N/10 NaOH neutralised.

A further set of analyses, see Table V, was made of the total globulin, and euglobulin of horse-serum, as well as the total protein precipitated by various methods. The specimens used were all prepared by Dr P. Hartley with great precautions and carefully purified and I am greatly indebted to him for generously placing this valuable material at my disposal. The results showed that none was free from phosphorus. In some cases the proportion contained showed great variation according to the method employed in preparation (*e.g.* nos. 1, 2, and 3, Table V). The euglobulin contained the greatest amount of phosphorus, and the content was about the same whether precipitated with ammonium sulphate or by dilution and acidification of the serum. This would indicate that the phosphorus contained in euglobulin was in closer association than the traces found present in the purified samples of the other proteins.

Any direct evidence regarding the character of the union of phosphorus in euglobulin is at present scanty, but the following results are worthy of consideration. Haslam [1913] was able to remove about half the total phosphorus from a sample of euglobulin by means of extraction with alcohol and ether. Absolute alcohol must, however, in case of proteins be regarded as a powerful reagent, causing serious changes; all are rendered permanently insoluble if contact is long enough and the temperature is allowed to rise above a low maximum. If previous to extraction with ether, acetone[1], in place of alcohol, is employed to remove the water, no damage appears to be suffered.

The material can then be extracted with ether, three or four changes being employed to remove completely all acetone, and the euglobulin remaining in contact with ether at room temperature for one or two days. The last ether extract being removed, the euglobulin is shaken up once more with ether, which is evaporated by pouring the euglobulin suspension on to a warmed porcelain basin and allowing a gentle current of warm air to play over it, the whole operation taking place in a hot room (about 36°). The extracted euglobulin is then obtained as a fine, white powder, which is exceedingly hygroscopic. The solvents employed should all be carefully purified and freed from water.

The extracted euglobulin was found to have lost nearly all its phosphorus; on one occasion it was reduced to less than one-quarter of the original (see Table V, original phosphorus-content 0·094 %; final 0·02 %). It is possible that the care taken to free all materials from water may account for the

[1] Acetone can be employed to remove water from the proteins of whole serum, without impairing their solubility in water.

large proportion of the phosphorus removed from the euglobulin in these experiments when compared with those of Haslam.

The ether-extracted euglobulin, like that treated by acetone, was readily soluble in water containing a small amount of acid or alkali. Its solubility in dilute salt solution was, however, always found to be reduced to a greater or less extent. On one occasion about 13 % was found to be soluble in dilute sodium chloride (0·85 %); in most cases the ether-extracted material was totally insoluble in salt solution.

The acetone and ether extracts, when taken to dryness, were found to contain fatty substances, presumably the lipoids contained in the original euglobulin. These fatty materials, which contained a considerable proportion of phosphorus, amounted in one instance to as much as 13·3 % by weight of the protein taken for the experiment. On one occasion, when the extracted euglobulin was rubbed up in a mortar with the fatty residue and salt solution, the power of salt solubility was found to have been restored to some extent, and a similar result was obtained with petrol-extracted euglobulin and the residue from the petrol extract. These two experiments were made with the same sample of euglobulin. With a second sample of euglobulin the experiment was unsuccessful and with a third a trace only was rendered soluble in dilute salt solution by this means. In the case of the first, successful, experiment a large proportion of the lipoid extracted from a comparatively large quantity of euglobulin was devoted to a small fraction of the treated protein and this procedure appears to be necessary to obtain any union and consequent restoration of salt solubility.

Some experiments with ether-extracted euglobulin were also made in which a lecithin emulsion was substituted for the fatty substance extracted from the euglobulin itself. In some instances the lecithin-treated material had distinctly greater solubility in sodium chloride, in others little or no difference from the control could be demonstrated. The question is still under investigation.

The evidence contained in the preceding paragraphs in favour of regarding euglobulin as a mechanical complex consisting of a protein (pseudo-globulin) and a lipoid may briefly be summarised as follows. The phosphorus-content is quite inconstant and shows wide variation in specimens prepared from different samples of serum. By extraction with acetone and ether, under certain circumstances, almost all the phosphorus can be removed together with a small amount of a fatty body. By this procedure the euglobulin, otherwise unchanged, loses its property of salt solubility, which characteristic

has, in some cases, been restored by addition of a lipoid to the ether-extracted material.

As regards the nature of this complex, euglobulin in serum must, in my opinion, be regarded as the product of the interaction of two colloidal systems, viz. the colloidal solution of pseudo-globulin and the emulsion of some lipoid present in the serum. The process is not reversible. The serum-lipoid remains firmly associated with the protein, and cannot be separated by repeated dispersion and re-precipitation, but can be removed by extraction with acetone followed by ether. The freed protein is in the "denaturated" condition, the pseudo-globulin having apparently undergone this irreversible change in the first instance under the influence of the lipoid.

Walpole [1914] has pointed out that, in some respects, euglobulin shows analogies with certain artificial colloidal systems which he has investigated, as, for example, solutions of gelatin to which suspensions of mastic or emulsions of oil have been added. In these mixtures of two colloidal constituents with widely separated iso-electric points, the dual nature of the system is apparent in the want of accurate coincidence between the iso-electric point and that of optimum flocculation. The same phenomenon has been demonstrated in the case of euglobulin [Chick 1913] and may doubtless be similarly explained. Feinschmidt [1912] determined the iso-electric point of six samples of lipoid from various sources to lie at a concentration of hydrogen-ions between about 10^{-2} and $10^{-3\cdot7}$ normal. The iso-electric point of the protein, on the other hand, is at a concentration of hydrogen-ions somewhere between 10^{-5} and 10^{-6} normal. [Michaelis and Rona 1910, 1, Michaelis and Davidsohn 1911, Sörensen and Jürgensen 1911, Chick and Martin 1912.]

The identification of the protein in euglobulin with pseudo-globulin is justified on chemical grounds by some recent work of Hartley[1]. He has shown that on examination of the three proteins (euglobulin, pseudo-globulin and albumin) of serum (horse and ox) by van Slyke's method, the composition of pseudo-globulin and euglobulin is found to be practically the same while a marked difference exists in case of serum albumin.

Summary.

1. Pseudo-globulin, prepared from diluted serum by precipitation with ammonium sulphate, the euglobulin having been previously removed as far as possible by dilution and bringing the solution to the iso-electric point,

[1] Communicated to the Physiological Society, Feb. 14, 1914 (Lister Institute, London) and to the Biochemical Society, July 11, 1914 (Oxford).

was always found, at the end of a long dialysis, to contain large quantities of a water-insoluble protein, resembling euglobulin.

2. Evidence is adduced to show that this insoluble material is not merely euglobulin present in the original serum and precipitated on dialysis, but is largely derived from the pseudo-globulin by a gradual process of "denaturation."

3. The gradual precipitation of the insoluble protein is accompanied by a disappearance from the solution of the small amount of phosphorus originally present in the pseudo-globulin preparation, after which there is no further separation of water-insoluble protein.

4. On addition of a watery emulsion of lecithin to thoroughly dialysed pseudo-globulin, from which insoluble protein and phosphorus had been removed as above, the "denaturation" process could be re-initiated.

5. The presence of minute amounts of acid and alkali or of a small concentration of salt (e.g. 1 % sodium chloride) prevents the precipitation of pseudo-globulin by a watery lecithin emulsion but, on dialysis, an insoluble protein, containing phosphorus and displaying the properties of euglobulin, is separated.

6. This artificial euglobulin appears to be a mechanical complex resulting from the interaction and mutual precipitation of the two colloidal systems: (*a*) the solution of pseudo-globulin and (*b*) the lipoid emulsion.

7. It is suggested that the euglobulin in serum is a protein-lipoid complex of similar origin. In support of this view the following facts are brought forward:

(*a*) The inconstant phosphorus-content.

(*b*) The readiness with which phosphorus can be removed from euglobulin by extraction with acetone and ether, during which process the property of salt solubility is lost. This can, in some cases, be restored by addition of the extracted fatty substance or of lecithin to the extracted euglobulin.

(*c*) The want of accurate coincidence between the iso-electric point of euglobulin and the point of optimum flocculation, as pointed out by Walpole [1914].

(*d*) The similarity in chemical composition between pseudo-globulin and euglobulin, as shown by Hartley.

In conclusion I desire to express my indebtedness to Prof. C. J. Martin, F.R.S. for valuable criticism.

BIBLIOGRAPHY.

Banzhaf (1911). *Johns Hopkins Bulletin* **32**, No. 241, 106.
Chick (1913). *Biochem. J.* **7**, 318.
—— (1914). *Biochem. J.* **8**, 59.
Chick and Martin (1910). *J. Physiol.* **40**, 404.
—— (1911). *J. Physiol.* **43**, 1.
—— (1912). *J. Physiol.* **45**, 261.
Feinschmidt (1912). *Biochem. Zeitsch.* **38**, 244.
Freund and Joachim (1902). *Zeitsch. physiol. Chem.* **36**, 407.
Gibson (1906). *J. Biol. Chem.* **1**, 161.
Handovsky and Wagner (1911). *Biochem. Zeitsch.* **31**, 32.
Hardy (1905). *J. Physiol.* **33**, 251.
Haslam (1913). *Biochem. J.* **7**, 492.
Maclean (1914). *J. Path. Bact.* **18**, 490.
Mellanby (1905). *J. Physiol.* **33**, 338.
Michaelis and Rona (1910, 1). *Biochem. Zeitsch.* **27**, 38.
—— (1910, 2). *Biochem. Zeitsch.* **28**, 193.
Michaelis and Davidsohn (1911). *Biochem. Zeitsch.* **33**, 456.
Sörensen and Jürgensen (1911). *Biochem. Zeitsch.* **31**, 397.
Walpole (1914). *Biochem. J.* **8**, 170.

XLVI. THE HYDROLYSIS OF GLYCOGEN BY DIASTATIC ENZYMES. Part III. FACTORS INFLUENCING THE END-POINT OF THE HYDROLYSIS.

By ROLAND VICTOR NORRIS, *Beit Memorial Research Fellow.*

(*From the Biochemical Laboratory, The Lister Institute, London.*)

(*Received July 14th, 1914.*)

In a previous communication [Norris, 1913, 1] the author confirmed the result previously obtained by Philoche [1908] that in the hydrolysis of glycogen by diastatic enzymes, the hydrolysis is as a rule far from complete, and also pointed out that the final degree of hydrolysis, as represented by the maltose production, increases with rising concentrations of enzyme though at a very much slower rate. This is therefore rather a different case from the breaking down of starch, where, although the hydrolysis is not complete, the equilibrium point according to most workers is the same with different concentrations of enzyme, viz. about 80·8 per cent. maltose and 19·2 per cent. dextrin. In this connection however Maquenne and Roux [1906, 1, 2, 3] suggest that the reaction only comes to an end owing to an insufficient activity of the enzyme and state that if the diastase be activated by the addition of traces of acid the hydrolysis proceeds to completion, a result which has been confirmed by Bayliss [1914].

It was also found by Brown [1904] that if the maltose formed were removed by fermentation with yeast the whole of the dextrin was hydrolysed, a result which suggested that the products exerted a retarding influence. As however acid is produced from the yeast this result may be really due to the activation of the enzyme and not to the removal of the products.

Under these circumstances it seemed of interest to examine the causes limiting the hydrolysis of glycogen by the glycogenase of the pancreas and some experiments carried out from this point of view are described in the present communication.

The glycogen used was prepared from oysters by Pflüger's method and

purified by repeated precipitation with alcohol. In those experiments where the influence of salts was under investigation the solutions were dialysed before use for several days against frequent changes of distilled water in presence of toluene.

The enzyme was as a rule prepared from dried pigs' pancreas which had previously been rendered fat free, by extraction with 50 per cent. alcohol and precipitation of the filtered extract with alcohol and ether. The precipitate was again taken up in 50 per cent. alcohol, filtered and reprecipitated, this process being repeated two or three times.

The course of the hydrolysis was followed by estimations from time to time of the reducing power of the solutions by Bertrand's method, the assumption being made that the reduction was due to maltose alone. While this is probably not entirely correct, the method is far more convenient than estimations of the optical rotation. The latter is open to the same objection as the former method and in addition necessitates the employment of very dilute solutions whereby further inaccuracies are in all probability introduced. In many cases the iodine reaction was also followed during the experiment.

Exp. 1. *Influence of enzyme concentration on the hydrolysis of glycogen.*

A. 105·5 cc. solution containing 1 g. glycogen, 0·1 cc. enzyme and 1 cc. toluene.

B. 105·5 cc. solution containing 1 g. glycogen, 0·5 cc. enzyme and 1 cc. toluene.

C. 105·5 cc. solution containing 1 g. glycogen, 5·0 cc. enzyme and 1 cc. toluene.

The above were incubated at 37° and samples taken from time to time for analysis, the results being shown in the following table.

TABLE I.

Duration of experiment Hours	Percentage hydrolysis		
	A.	*B.*	*C.*
1	36·65	52·90	69·8
2	40·45	54·00	77·1
3	42·80	54·30	82·0
4	43·70	54·55	84·8
5	44·60	54·70	87·4
6	45·10	—	89·0
7	45·40	—	90·2
8	45·60	—	91·0
9	45·60	55·10	91·5

In the above experiment it will be seen that while with the highest concentration of enzyme rather over 90 per cent. of the glycogen was completely hydrolysed to maltose, with 0·1 cc. enzyme only about half this quantity of maltose was formed. At the same time all the glycogen had disappeared, having been converted into a dextrin which remained to a large extent unhydrolysed. The intermediate products in the hydrolysis of glycogen have not received a great amount of attention. Tebb [1898] however found that a dextrin was formed which was extremely resistant to further hydrolysis and identified this with the dextrin previously described by Seegen [1879] and termed by him dystropodextrin. This dextrin gives no iodine reaction, in fact with pancreatic glycogenase no erythrodextrins appear to be formed, or if they are produced they are hydrolysed so rapidly that it is impossible to detect them.

It is difficult to believe however that the resistant nature of this dextrin is the cause of the stopping of the hydrolysis. For considering the experiment just described, the rates fell off when the reduction indicated a hydrolysis of rather over 40 per cent. in *A*, of 50 per cent. in *B*, and over 80 per cent. in *C*. In other words the slower the initial rate of hydrolysis, the greater was the quantity of resistant dextrin apparently formed. In the case of *C* the slowing off in the rate could be quite well explained by the exhaustion of the substrate, but in *A* and *B* this explanation cannot hold. Another possible solution is that two enzymes may be concerned. This view in the case of diastase has been advanced by several investigators [Brown and Heron 1879; Kjeldahl 1879; Duclaux 1899; Fraenkel and Hamburg 1906]. Now if the enzyme preparation employed in these experiments consists of a large proportion of glycogenase which only carries the hydrolysis to the dextrin stage and a very small amount of dextrinase the results obtained above are to a certain extent what would be expected. For in this case with the low enzyme concentration there might be enough glycogenase to carry out the first stage of the hydrolysis but insufficient dextrinase to hydrolyse the dextrin thus formed at a corresponding rate. In this way an accumulation of dextrin would take place. With the high concentration of enzyme the rate of the first stage of the hydrolysis would be limited by the concentration of glycogen employed and hence would not increase proportionally with rising enzyme concentrations. The second stage of the hydrolysis however would proceed at a much greater rate and hence the accumulation of dextrin would be less as the concentration of enzyme increased. Now while this is actually the final result found experimentally an examination of Table I shows that

when the slow stage of the hydrolysis is reached, *B* and *C* are proceeding not very much more quickly than *A*. Hence some other factors must be concerned and the two which first suggest themselves are (1) destruction of the enzyme during the course of the experiment, and (2) retarding influence of the products. Before considering these points however it will be as well to describe some further experiments in which the results indicated above were reproduced by other methods.

It is well known that the activity of a glycogenase preparation is almost completely lost on dialysis but can be restored by the addition of certain salts, notably chlorides. A certain minimum concentration of such salts varying with the concentration of enzyme is required to produce the optimum rate of hydrolysis [Cole 1906; Starkenstein 1910, 1; Norris 1913, 2].

Now if a series of dialysed glycogen solutions is made up, each containing the same quantity of dialysed glycogenase but increasing quantities of KCl, not only does the initial rate of hydrolysis increase with rising concentration of salt but the final degree of hydrolysis also rises. These results only obtain of course until the salt concentration reaches the optimum required for the particular strength of enzyme employed.

Exp. 2. *Influence of salt content on the total degree of hydrolysis.*

A. 100 cc. 1 per cent. dialysed glycogen containing 1 cc. toluene and 0·5 cc. dialysed enzyme.

B. 100 cc. 1 per cent. dialysed glycogen containing 1 cc. toluene, 0·5 cc. N/20 KCl and 0·5 cc. dialysed enzyme.

C. 100 cc. 1 per cent. dialysed glycogen containing 1 cc. toluene and 2·0 cc. N/20 KCl and 0·5 cc. dialysed enzyme.

The solutions were incubated at 37° and the reducing power estimated at frequent intervals.

In this experiment *A* which contained no added salt had still a low activity, the dialysis not having been carried sufficiently far to render the solution entirely salt free. *A* therefore represents a solution with very low salt content.

Results :

TABLE II.

Duration of experiment Minutes	Percentage hydrolysis		
	A.	*B.*	*C.*
15	2·07	9·2	13·2
30	3·22	14·6	22·7
60	5·07	21·6	—
75	—	—	30·18
120	10·14	25·68	—
135	—	—	33·71
240	12·89	28·4	—
255	—	—	37·44
360	—	29·28	—
420	16·58	—	38·34
480	—	29·73	39·24

The results are therefore exactly similar to those obtained when different concentrations of enzyme are employed and indicate that a certain amount of the enzyme remains entirely inactive in presence of insufficient salt. This is of some interest as Starkenstein [1910, 2] has shown that even in the absence of any salt, adsorption of diastase by starch still takes place though no chemical change results until salt is added. The salt presumably must have some specific activating effect on the enzyme and its only function cannot be to assist the combination between enzyme and substrate. It also shows that if two enzymes are present they are equally dependent on the presence of salts.

In view of the above experiment it was of interest to see what would be the effect of adding further salt to a solution such as *A* or *B* above when the rate of hydrolysis had become stationary and hence the following experiment was carried out.

Exp. 3. *Addition of salt at "stationary point."*

A. 50 cc. 2 per cent. glycogen + 0·25 cc. N/20 KCl + 1 cc. toluene + 0·5 cc. enzyme + 48·25 cc. H_2O.

B. 50 cc. 2 per cent. glycogen + 10 cc. N/20 KCl + 1 cc. toluene + 0·5 cc. enzyme + 38·5 cc. H_2O.

C. As *A*.

The glycogen and enzyme solutions were dialysed before use. When the rate of hydrolysis in *A* and therefore in *C* had become extremely slow, further salt was added to *C* so that the concentration of KCl became equal to that in *B*.

The course of the hydrolysis in each case is shown in Table III below.

TABLE III.

Duration of experiment Minutes	Percentage hydrolysis		
	A. Low salt content	*B.* High salt content	*C.* Low salt content. Salt added at stationary point
15	3·95	10·14	—
30	7·39	17·40	7·39
240	24·80	33·50	24·90
315	26·50	35·20	26·54
			KCl added here
345	26·80	35·80	28·60
405	27·50	—	31·40
465	27·70	—	32·32
585	28·30	37·80	34·16
705	28·78	38·20	34·63

The results show that the addition of salt to *C* after 315 minutes resulted in an increase in the rate of hydrolysis, *C* increasing 8·1 per cent. during the remainder of the experiment as against the 2·2 per cent. increase shown by *A*. Compared with *B* however, which contained the high salt concentration all the time, *C* still showed a deficit of 3·6 per cent. when the two rates had become equal. This however would be expected as even assuming that all the excess of enzyme not combined with salt were intact when further salt was added, there was at this point already present in *C* a considerable percentage of the hydrolytic products which might retard the freshly activated enzyme. At the same time if the curves shown in Fig. 1 representing this experiment be observed it will be noticed that the rate of action in *C* after the addition of the salt was slightly less than that obtaining in *B* when at the same stage of the hydrolysis, this suggesting that slight injury to the enzyme had occurred.

The case when further enzyme is added at the stationary point is also of interest and will be considered next.

Exp. 4. *Addition of enzyme at "stationary point."*

A. 50 cc. 2 per cent. dialysed glycogen + 0·25 cc. N/20 KCl + 1 cc. toluene + 0·5 cc. dialysed enzyme.

D. 50 cc. dialysed 2 per cent. glycogen + 10 cc. N/20 KCl + 1 cc. toluene + 2 cc. dialysed enzyme.

E. 50 cc. 2 per cent. dialysed glycogen + 0·25 cc. N/20 KCl + 1 cc. toluene + 2 cc. enzyme.

F. As *A*. Enzyme added at stationary point so that enzyme concentration then became equal to *E*.

G. As *A*. Enzyme and salt added at stationary point so that the mixture was then similar in composition to *D*.

The hydrolyses were carried out in the usual manner till *F* and *G* had become very slow, which occurred after about five hours, when the additions were made, the results being shown in Table IV.

TABLE IV.

	Percentage hydrolysis				
Duration of experiment minutes	*A.* Low salt, low enzyme content	*D.* High salt, high enzyme content	*E.* Low salt, high enzyme content	*F.* Addition of enzyme	*G.* Addition of enzyme + salt
300	26·2	42·1	36·0	26·2	26·2
					Enzyme and salt added
315	26·5	42·4	36·2	26·54	—
				Enzyme added	
330	26·65	42·6	36·3	—	32·32
345	26·80	42·8	36·4	29·36	—
390	27·30	—	—	—	37·90
405	27·50	—	—	31·86	38·50
585	28·30	43·15	37·8	35·11	41·60
705	28·78	43·69	38·3	35·60	42·05

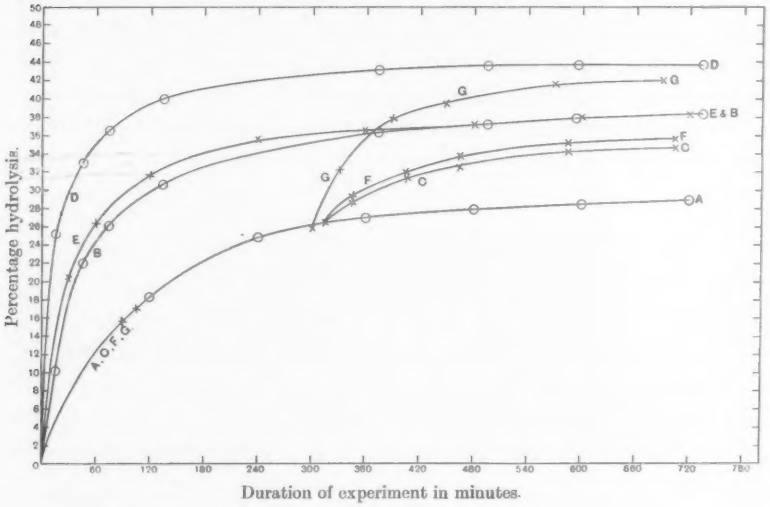


Fig. 1.

The first point of interest is that addition of further enzyme to *F* produced an acceleration although the concentration of salt employed was quite insufficient to permit the enzyme initially present to produce its maximum

effect. There is evidently then an equilibrium established between free salt, free enzyme and the salt-enzyme complex which is changed by the addition of further enzyme, more of the complex being formed and the hydrolysis thereby accelerated. The same point is indicated by the two controls *A* and *E*, *E* proceeding at a much greater rate than *A* although having the same low salt content. The results are shown graphically in Fig. 1, together with those of Experiment 3.

The addition of salt and enzyme naturally produces a greater acceleration than the addition of either separately, and this acceleration is in fact greater than the sum of the two components as one would expect, the enzyme in *F* being retarded by an insufficiency of salt. At the same time the rate in *G* after the addition is less than that obtaining in the control *D* when at the same stage of the hydrolysis. Examining the total degree of hydrolysis produced it will be seen that while *G* never quite reached the control *D*, it approached it very nearly and this also applies to *F* and its control *E*, the deficiency being however slightly greater in this case.

All these results are against the view that the stopping of the action is caused by the resistance of the residual dextrin to further hydrolysis, the addition of further enzyme producing initially a fairly rapid rate of hydrolysis which quickly falls off again, thus suggesting an alteration of an equilibrium between enzyme and the hydrolytic products. The only other obvious factor is the possible destruction of the enzyme during the course of the experiment and the degree to which this is concerned was examined in the following experiment.

Exp. 5. *Addition of further glycogen at the "stationary point."*

A. 100 cc. 1 per cent. glycogen + 1 cc. toluene + 1 cc. enzyme.

B. 100 cc. 2 per cent. glycogen + 1 cc. toluene + 1 cc. enzyme.

C. Initially as *A*. Further glycogen added after 4 hours hydrolysis.

Hydrolysis was allowed to proceed at 37° until the rate in *A* and *C* became slow and then further glycogen was added to *C* so that the total concentration became equal to that originally present in *B*, and the further course of the hydrolysis observed.

The results are shown graphically in Fig. 2, the abscissae representing the duration of the experiment in minutes and the ordinates the amount of hydrolysis which had taken place expressed in percentages of one gram, the latter being calculated as previously explained on the assumption that the reducing power of the solution is due to maltose alone.

The glycogen was added to *C* at 240 minutes and as will be observed was rapidly hydrolysed, showing that a large proportion of the enzyme was still in an active condition although the rate was much lower than that at which the first quantity of glycogen was initially hydrolysed, the relative values for 60 minutes being 9·5 and 17 respectively. As the final degree of hydrolysis observed was practically the same however in *B* and *C* it is extremely improbable that any of the enzyme had been destroyed during the experiment and the difference in the two rates must be attributed to the influence of the products present when the addition of glycogen was made. This view is supported by the next experiment.

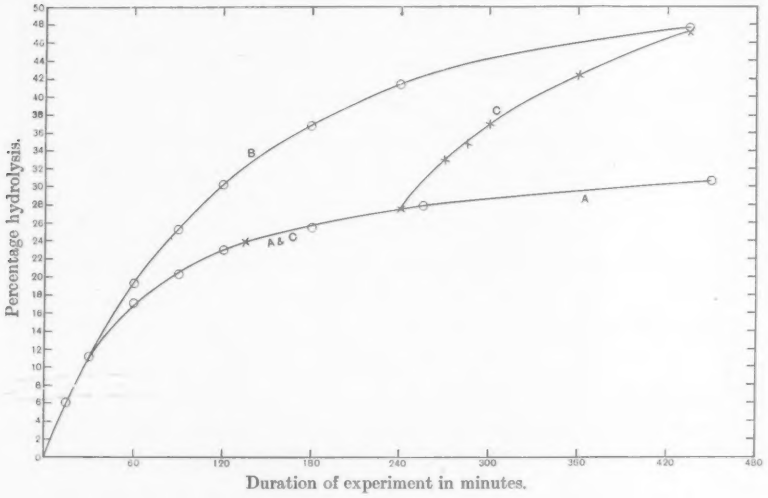


Fig. 2.

Exp. 6. *Addition of glycogen and enzyme at the "stationary point."*

A. 100 cc. 1 per cent. glycogen + 1 cc. enzyme + 1 cc. toluene.

B. 100 cc. 2 per cent. glycogen + 2 cc. enzyme + 1 cc. toluene.

C. Initially as *A*. When the action had slowed down, enzyme and glycogen were added to make the proportion of these similar to those obtaining in *B*.

It was to be expected that the glycogen added to *C* would be hydrolysed with a considerable initial velocity but that this rate would fall off quickly owing to the influence of the products on the freshly added enzyme. For the

same reason it was unlikely that the final degree of hydrolysis would be as great in *C* as in *B*.

An examination of the results shown in Fig. 3 indicates that these hypotheses were verified. Here again the ordinates represent the degree of hydrolysis expressed in the percentages of one gram hydrolysed to maltose.

The glycogen and enzyme were added to *C* after 300 minutes and while the glycogen was readily hydrolysed the rate was actually less than that obtaining initially in *A* which had only half the concentration of enzyme. This result can only be explained by the influence of the products as whatever

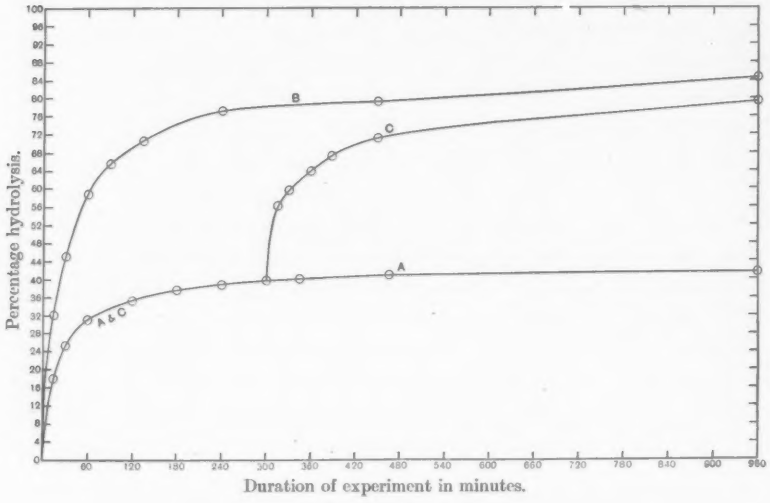


Fig. 3.

may have happened to the first quantity of enzyme during the course of the experiment, that added later was in an active condition and yet could not give such a high rate of hydrolysis as a similar quantity of enzyme acting on a solution containing none of these products. Doubtless for the same reason the final degree of hydrolysis in *C* was less than that observed in *B*.

Influence of the hydrolytic products. (*a*) *Maltose.*

Exp. 7. *A.* 100 cc. 1 per cent. glycogen solution + 1 cc. toluene + 0·5 cc. enzyme.

B. 100 cc. 1 per cent. glycogen solution + 1 cc. toluene + 0·5 g. maltose + 0·5 cc. enzyme.

The only difference between the two experimental mixtures is that *B* contains initially an amount of maltose corresponding to that found at the conclusion of a hydrolysis with this strength of enzyme. The rates at which hydrolysis proceeded in *A* and *B* were then compared, the results being shown in Table V.

TABLE V.

Duration of experiment	mg. Cu from 20 cc. solution	
	A.	*B.* Corrected for added maltose
15 mins.	27	28
30 „	46	46
1 hr.	66	65
2 hrs.	80	80
4 „	92	91
8 „	100	99
20 „	108	104
Percentage hydrolysis	49·6	47·6

This quantity of maltose had then very little depressing action on the rate of hydrolysis.

(*b*) *Influence of mixed products.*

Exps. 8 and 9. 200 cc. of 1 per cent. glycogen solution containing 2 cc. enzyme and toluene were incubated at 37° for 24 hours. The solution was then boiled and the degree of hydrolysis accurately determined.

The following experiments were then carried out.

A. 100 cc. 1 per cent. glycogen solution + 1 cc. toluene + 1 cc. enzyme.

B. 100 cc. boiled solution containing hydrolytic products + 1 g. glycogen + 1 cc. toluene + 1 cc. enzyme.

Two similar experiments were carried out, the results being shown in Table VI.

A control was carried out in which the boiled solution of products alone was incubated with enzyme but practically no further change took place during the course of the experiment.

In Experiment 9 there was a slightly higher initial proportion of products than in 8. In both cases however there is a well marked retarding influence exerted by the hydrolytic products, the differences observed at the end of the experiment being as follows:

Exp. 8. 5·69 in a hydrolysis of 46·55 = 12·2 per cent.

Exp. 9. 6·26 in a hydrolysis of 47·10 = 13·2 per cent.

It is possible that the freshly formed products may have an even greater influence than those examined in the experiments where the solutions had been boiled.

TABLE VI.

Duration of experiment Minutes	Experiment 8		Experiment 9	
	Percentage hydrolysis			
	A.	*B*.	A_1.	B_1.
15	21·40	19·0	18·0	15·2
30	29·49	26·6	27·1	21·87
60	33·77	29·55	33·28	27·65
120	38·57	33·37	38·57	32·78
175	—	—	39·03	33·35
240	40·93	34·3	—	—
360	42·78	36·2	—	—
510	43·25	37·15	—	—
675	—	—	43·72	37·9
1375	—	—	47·1	40·84
1380	46·55	40·86	—	—

Summary and Conclusions.

1. A solution of glycogen is not completely hydrolysed to maltose by the glycogenase of the pancreas unless very high concentrations of enzyme are employed, although, even with low enzyme concentrations, the whole of the glycogen may disappear.

2. The total degree of hydrolysis produced rises with increasing concentration of enzyme but not proportionally.

3. In dialysed solutions the same result is obtained by increasing the salt content up to a certain optimum concentration.

4. Evidence is brought forward to show that the resistance of the residual dextrin to further hydrolysis is not the main factor in the stopping of the reaction.

5. There is little evidence indicating that destruction of the enzyme takes place to any considerable extent during the course of the experiment.

6. The mixed products formed have a marked retarding influence on the velocity of the reaction and are probably the most important factors in the stopping of the hydrolysis. Maltose alone however has a very small effect.

REFERENCES.

Bayliss (1914), Nature of Enzyme Action, 3rd Edition, London, 97.
Brown, A. J. (1904), Laboratory Studies for Brewing Students London, 105.
Brown and Heron (1879), *J. Chem. Soc.* **35**, 596.
Cole (1906), *J. Physiol.* **30**, 202.
Duclaux (1899), Traité de Microbiologie, Tome II, Paris, 392.
Fraenkel and Hamburg (1906), *Beiträge*, **8**, 389.
Kjeldahl (1879), *Compt. Rend. Carlsberg*, Abstract *Pharm. J.* 1910, 362.
Macquenne and Roux (1906, 1), *Compt. Rend.* **142**, 124.
—— (1906, 2), *Compt. Rend.* **142**, 1059.
—— (1906, 3), *Compt. Rend.* **142**, 1387.
Norris (1913, 1), *Biochem. J.* **7**, 26.
—— (1913, 2), *Biochem. J.* **7**, 622.
Philoche (1908), *J. Chim. Phys.* **6**, 212, 355.
Seegen (1879), *Pflüger's Archiv*, **19**, 106.
Starkenstein (1910, 1), *Biochem. Zeitsch.* **24**, 191.
—— (1910, 2), *Biochem. Zeitsch.* **24**, 210.
Tebb (1898), *J. Physiol.* **22**, 423.

XLVII. ON THE PURINE METABOLISM OF RATS.

By HAROLD ACKROYD.

(*From the Institute for the Study of Animal Nutrition, Department of Agriculture, Cambridge.*)

(*Received July 15th, 1914.*)

Folin and Morris [1913] make the statement that "The purine metabolism of rats is like that of man and unlike that of other mammals hitherto investigated." If this were true the rat would be a most valuable animal on which to investigate the problems of gout.

This statement rests on the following evidence:

1. Rats excrete about 0·5 mg. of uric acid nitrogen a day. This *in proportion to body weight* is about as much as is found in human urine.

2. 100 g. of rat's blood contains 2·4–2·5 mg. of uric acid, "substantially the same figures as Folin and Denis found for human blood."

3. From 50 cc. of rat's urine enough uric acid was isolated for complete identification.

No attempt was made to estimate the allantoin in rat's urine or to observe the effect on the purine and allantoin excretion of adding purine substances to a purine free diet.

The essential difference between the purine metabolism of man and other mammals, except perhaps the chimpanzee, is not in the amount of uric acid excreted in proportion to body weight or the percentage of it contained in the blood, but rather in the fact that in man the purines are not further converted to allantoin as an end product while in other mammals this is the case. The observations recorded in the experimental part of this paper show that the relatively large amount of uric acid excreted by rats is an expression of a very high rate of purine metabolism, the main end product of which is allantoin, this latter representing about 15 times as much nitrogen as that of the total purine bases and uric acid.

Moreover when hypoxanthine is given by the mouth to rats about 60 per cent. of its nitrogen is excreted in the form of allantoin while only about 3 per cent. is excreted as purine base + uric acid nitrogen. This latter observation is also of interest from the fact that Jones and Rhodé [1909] were unable to demonstrate the existence in rat's organs of a ferment capable of destroying hypoxanthine.

Experimental Part.

The rats were kept in pairs in a modified form of the cage described by Völtz [1912]; the urine and faeces were separated by the ball method originally used at the Cancer Hospital, Fulham.

The cages were washed down daily and the urine + washings from each pair of rats preserved with toluene for seven days. The toluene was driven off on the water bath and the seven days' urine made up to 500 cc.

5 cc. were used for total nitrogen by Kjeldahl's method, 275 cc. for purine base + uric acid nitrogen by the Camerer-Arnstein method and 200 cc. for allantoin by Wiechowski's method. In this latter the original method was carried out exactly as described [Wiechowski 1907], and the figures for allantoin nitrogen, calculated from the weight obtained, as well as those given by the Kjeldahl analysis of the mercury acetate precipitate, are given. Most writers seem to consider the latter more accurate and I am inclined to agree with this view from subsequent observations when the diet was less simple in character than bread and water. The allantoin obtained melted in every case at 232°–234°; its solution was not precipitated by silver nitrate but gave a copious precipitate on further addition of ammonium hydrate, soluble in excess of the latter. Boiled with caustic soda it gave the glyoxylic reaction. A collection of the allantoin obtained once recrystallised from water gave the following analysis:

Found	N = 35·0 %	Calculated	N = 35·44 %
	C = 29·73 %		C = 30·3 %
	H = 3·82 %		H = 3·8 %

The rats were fed on white bread and water for two weeks before and throughout the experiments. The amount of bread eaten was not limited, for although white bread contains traces of allantoin [Ackroyd 1911] the quantity in the bread eaten by a rat is negligible in comparison with the amount excreted.

All the figures given are calculated as excretion of one rat for one day and represent an average of seven days' excretion for two rats.

During the second week 0·45 g. of hypoxanthine was given to each pair of rats; this was given in equal portions with the first food in the morning of the first five days of the period, the last two days were intended to cover any delayed excretion. This would be equivalent to 0·013 g. purine nitrogen per rat per day calculated for the whole seven day period.

Rats A. Two males weighing 158 *g. and* 174 *g.*

Date	Total N mg.	Purine N mg.	Allantoin N mg. by weight	Allantoin N mg. by Kjeldahl	Diet
Jan. 19–26	191	0·62	10	9·5	Bread, water
Jan. 26–Feb. 2	182	0·95	18·3	17·4	Bread, water. 13 mg. hypoxanthine N
Feb. 2–9	137	0·51	9·4	8·7	Bread, water
Feb. 9–16	156	0·5	8·7	8·5	Bread, water

The average excretions of the first, third and fourth periods are: purine nitrogen 0·54 mg.; allantoin nitrogen 8·9 mg. The increased excretions over these averages in the second period are 0·41 mg. purine N and 8·5 mg. allantoin N equivalent to a recovery of 3·1 % as purine N and 65 % as allantoin N of the extra hypoxanthine N given.

Rats B. Two males weighing 133 *g. and* 165 *g.*

Date	Total N mg.	Purine N mg.	Allantoin N. mg. by weight	Allantoin N. mg. by Kjeldahl	Diet
Jan. 20–27	172	0·66	8·7	8·5	Bread, water
Jan. 7–Feb. 3	191	1·06	17·4	16·3	Bread, water 13 mg. hypoxanthine N
Feb. 3–10	180	0·6	9·8	10	Bread, water
Feb. 10–17	145	0·63	7·5	7·7	Bread, water

The average excretions of the first, third and fourth periods are purine N 0·63 mg., allantoin N 8·4 mg. The increased excretions over these averages in the second period are: purine N 0·43 mg., allantoin N 7·9 mg. equivalent to a recovery of 3·3 % as purine N and 60·7 % as allantoin N of the extra hypoxanthine N given.

Conclusion.

The purine metabolism of rats is like that of other lower mammals and unlike that of man[1].

REFERENCES.

Ackroyd (1911), *Biochem. J.* **5**, 400.
Folin and Morris (1913), *J. Biol. Chem.* **14**, 509.
Jones and Rhodé (1909), *J. Biol. Chem.* **7**, 237.
Völtz (1912), Abderhalden, Handbuch der biochemischen Arbeitsmethoden, **5**, 1044.
Wiechowski (1907), *Beiträge*, **11**, 109.

[1] Since this work was finished (February 1914), Hunter and Givens (Proceedings of the American Society of Biological Chemists, *J. Biol. Chem.* **17**, xxiii) published an analysis of rats' urine; they find that of the purine + allantoin nitrogen 93 per cent. is present as allantoin, 4 per cent. as uric acid and 3 per cent. as purine bases.

XLVIII. THE PRODUCTION OF ω-HYDROXY-*s*-METHYLFURFURALDEHYDE FROM CARBOHYDRATES AND ITS INFLUENCE ON THE ESTIMATION OF PENTOSANS AND METHYLPENTOSANS.

By MARY CUNNINGHAM and CHARLES DORÉE.

(*From the Chemistry Department, Borough Polytechnic Institute, S.E.*)

(*Received July 13th, 1914.*)

In the method for the estimation of pentosans devised by Kröber and now extensively in use, the substance is boiled with a 12 per cent. solution of hydrochloric acid. The conditions are regulated so that 30 cc. are distilled over every fifteen minutes, 30 cc. of fresh acid being then added to the contents of the flask. The furfuraldehyde contained in the distillate is precipitated by the addition of phloroglucinol, and from the weight of the greenish black precipitate obtained the amount of furfuraldehyde, and consequently of pentosan present, may be calculated with the help of tables given by Kröber [1901]. In the case of methylpentosans, methylfurfuraldehyde is obtained which gives a red precipitate with phloroglucinol. This, unlike the black compound given by furfuraldehyde, is easily soluble in alcohol and on this difference Ellet and Tollens [1905] have based a method for the separation of the two phloroglucides and the consequent estimation of pentosan and methylpentosan groupings.

The phloroglucinol method has long been recognised as a "conventional" one in that (i) a definite procedure of working must be followed, (ii) the relation of the phloroglucide formed to pentosan present is empirical, (iii) other substances may be present in the distillates which also give a precipitate with phloroglucinol.

The difficulty last mentioned was investigated by Unger [1902], who noticed that various pure substances, and also natural products which contained no pentosan groupings, such as dextrose and starch, yielded, on

distillation with hydrochloric acid, volatile substances which could be precipitated with phloroglucinol. Tollens [1902] determined the percentage of "pentosan" given in this way by substances expected to contain very few or no pentosan groupings as follows:

	Per cent.		Per cent.
Cane sugar	1·2	Filter paper	1·5
Potato starch	0·8	Cotton wool	1·6

Grund [1902] also in an investigation of the content of "combined pentoses" in various animal tissues obtained an orange red precipitate from hexoses and from glycogen, and remarks that "apparently with hexoses in addition to furfuraldehyde, another and, till now, unknown body is formed." The complications brought about by the presence of these unknown substances are mentioned in many researches dealing with technical applications of the phloroglucinol method. Reed and Schubert [1914] for example, criticising methods in use for the testing of tanning materials, mention that the red phloroglucide precipitate indicating methylfurfuraldehyde given by various substances which are capable of yielding rhamnose on hydrolysis, such as xanthorhamnin, hesperidin, etc., is not absolutely reliable as a test for these, since dextrose gives a very similar, brown coloured precipitate.

A series of experiments described below has led us to the conclusion that, in the majority of cases, the substance giving this brown precipitate and the superficial reactions of methylfurfuraldehyde is ω-hydroxy-*s*-methylfurfuraldehyde, and that it is a constant product of the action of dilute hydrochloric acid on hexoses and on polyoses, such as starch and cellulose, which are capable of yielding hexoses on hydrolysis.

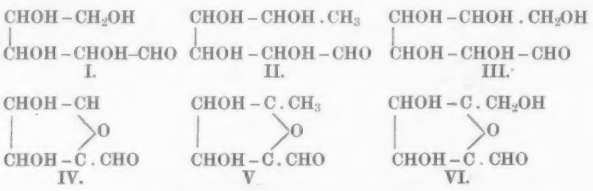
Method of Experiment.

The Kröber method prescribes that the distillation is finished when a drop of the liquid gives no red colour when brought on to aniline acetate paper. As methylfurfuraldehyde does not give this reaction, a solution of phloroglucinol in hydrochloric acid is used when methylpentosans are to be estimated. A solution of aniline in alcohol is also a very sensitive reagent for the detection of furfuraldehyde and its derivatives, and in some estimations we made use of it. We found however that the rose colour given by aniline-alcohol persisted in portions of the distillate long after the aniline acetate paper had ceased to indicate the presence of furfuraldehyde. As is well known in the case of vegetable tissues which contain a considerable proportion of pentosan groupings, the formation and elimination of the furfuraldehyde

are comparatively rapid, *e.g.* with 2 grams of jute (which yielded 9 per cent. of furfuraldehyde) 210 cc. of distillate were sufficient. After this stage no colour with aniline acetate could be obtained. A further 950 cc. however were distilled over before the colouration with aniline-alcohol had ceased to be given.

A number of experiments on these lines was therefore carried out on cellulose and other carbohydrates (see Table III). In each case the aniline-alcohol reaction was obtained during a more or less prolonged period of distillation. In some instances the aniline acetate test for furfuraldehyde could not be observed, in others it was possible to separate the distillate into (*a*) a portion giving the reaction with aniline acetate and containing furfuraldehyde, and (*b*) a portion reacting only with aniline-alcohol.

This second portion in every case gave several of the tests prescribed as characteristic of methylfurfuraldehyde, including the absorption spectrum test devised by Oshima and Tollens [1901] for the detection of this aldehyde in the presence of furfuraldehyde, and a brown precipitate with phloroglucinol soluble in alcohol. Seeing however that pentoses (I) and methylpentoses (II) readily yield furfuraldehyde (IV) and methylfurfuraldehyde (V) respectively, it seemed more probable that hexoses (III) would yield ω-hydroxymethylfurfuraldehyde (VI) thus :



van Ekenstein and Blanksma [1910] have already shown that this substance is formed by the action of dilute oxalic acid, under pressure, on hexoses, and they have proved that many of the colour tests given by the hexoses are due to the formation of ω-hydroxymethylfurfuraldehyde and not to furfuraldehyde as previously supposed. We therefore made a careful comparative examination of the hydrochloric acid distillates obtained from dextrose, starch, cellulose and lignocellulose and satisfied ourselves that these contained besides furfuraldehyde small quantities of ω-hydroxymethylfurfuraldehyde. This derivative is attacked by dilute acids, being converted into laevulic and formic acids thus :

$$C_6H_6O_3 + 2H_2O = C_5H_8O_3 + H \cdot COOH,$$

so that no very large proportion of the theoretical yield could be expected. A recent note of E. Fischer [1914] shows, however, that if the action of the acid be controlled and stopped before this secondary decomposition has begun, comparatively large yields of ω-chloromethylfurfuraldehyde may be obtained.

PROPERTIES AND REACTIONS OF FURFURALDEHYDE AND ITS DERIVATIVES.

For purposes of comparison methylfurfuraldehyde was prepared from rhamnose [Fromherz, 1906] and ω-hydroxymethylfurfuraldehyde both by the action of oxalic acid on cane sugar [Kiermayer, 1895] and from the chloro-derivative obtained from laevulose by Fenton's reaction. Our observations made from distillates from about 0·2 gram of each aldehyde are given in the following table.

TABLE I.

Reactions of furfuraldehyde and its derivatives.

	Furfuraldehyde	Methyl-furfuraldehyde	ω-Hydroxymethyl-furfuraldehyde
1. Aniline acetate	pink	yellow	pink
2. Aniline-alcohol	bright red	yellow-red	bright red
3. Phloroglucinol-ppt.	black	red	red-brown
4. ,, -filtrate ..	green	red-brown	orange-red
5. Absorption spectrum of (4) ..	none	band in blue green	band in blue green
6. α-Naphthol + H_2SO_4*	grey red greenish	colourless scarlet greenish	violet crimson emerald green
7. Thymol in alcohol + H_2SO_4 ..	red, violet above	red-pink, yellow above	scarlet with violet above
8. Barbituric acid	yellow ppt.	yellow ppt.	No ppt.

* Colours given in order from the top.

The second portion of the distillates from all the substances mentioned in Table IV gave reactions indistinguishable from those shown by ω-hydroxymethylfurfuraldehyde. The Maquenne test for methylfurfuraldehyde was obtained only with rhamnose. In order further to confirm the presence of the hydroxyaldehyde 64 grams of viscose silk were boiled under a reflux for 2 hours with 600 cc. of the hydrochloric acid. The filtered liquid was neutralised and extracted with ether. From the extract a brown oil giving the reactions of furfuraldehyde was obtained. The extracted water solution was distilled in a current of steam and gave a distillate which answered all the tests for ω-hydroxymethylfurfuraldehyde. This observation is in keeping with the fact that this substance is not extracted by cold ether from aqueous solution.

The Composition of the Precipitates given by Phloroglucinol with Furfuraldehyde and its Derivatives.

Kröber [1901] stated that the phloroglucide was formed by the condensation of one molecule of furfuraldehyde and one molecule of the phenol with elimination of two molecules of water thus:

$$C_5H_4O_2 + C_6H_6O_3 - 2H_2O = C_{11}H_6O_3,$$

and that the composition of the precipitate (C = 71·0; H = 3·2 per cent.) was independent of the conditions of precipitation. Tollens and Goodwin [1904] showed that the compound is considerably oxidised on drying in the air, but that preparations dried in hydrogen gave figures agreeing exactly with those required by the equation:

$$C_5H_4O_2 + C_6H_6O_3 - H_2O = C_{11}H_8O_4.$$

Assuming that a similar reaction takes place with the derivatives we have the following theoretical values:

TABLE II.

	Phloroglucide of:	Formula	requires
(I)	Furfuraldehyde	$C_{11}H_8O_4$	C = 64·7; H = 4·0
(II)	Methylfurfuraldehyde	$C_{12}H_{10}O_4$	C = 66·0; H = 4·6
(III)	Hydroxymethylfurfuraldehyde	$C_{12}H_{10}O_5$	C = 61·5; H = 4·2

The alcohol soluble part of a phloroglucide which we obtained from dextrose, after drying as far as possible with exclusion of air, gave results agreeing to some extent with the composition of the hydroxymethyl derivative:

0·1260; 0·2856 CO_2; 0·0550 H_2O.
Found C = 61·8; H = 4·3 per cent.

The formula $C_{12}H_{10}O_5$ gives the factor 0·54 for conversion of the weight of phloroglucide to weight of hydroxymethylfurfuraldehyde. In calculating Table III, the factor 0·6 was employed.

Quantitative Experiments.

In order to ascertain whether the constant formation of hydroxymethylfurfuraldehyde involves any error in the estimation of furfuraldehyde by the phloroglucinol method, and to obtain an idea of the amount produced, the experiments summarised in the table below were made. The precipitate given by that portion of the distillate which reacted to aniline acetate, if it could be collected separately, or if not, the portion of the total precipitate

insoluble in alcohol, was calculated as furfuraldehyde. The soluble portion was calculated as ω-hydroxymethylfurfuraldehyde (factor 0·6). A comparative value only can, however, be attached to these latter numbers. For the purification of the celluloses employed see Dorée [1913].

TABLE III.

No.	Substance	Weight (anhydrous)		Distillate cc.*	Weight of phloroglucide	Weight soluble in alcohol	Furfuraldehyde per cent.	ω-hydroxymethylfurfuraldehyde per cent.	Remarks
1.	Rhamnose	0·424		760	0·2630	all	—	—	Ppt. red and consists of methylfurfural phloroglucide
2.	Dextrose	2·123		610	0·0565	0·0550	0·18	1·6	No aniline acetate reaction; ppt. brown
3.	Laevulose	3·770		240	0·0843	0·0712	0·20	1·1	After 60 cc. no aniline acetate reaction; ppt. red
4.	Starch (potato)	3·774		680	0·0981	0·0902	0·15	1·4	After 250 cc. no aniline acetate reaction; ppt. red
5.	Cellulose (cotton wool)	2·021	(*a*)	100	0·0073	none	0·20		Ppt. black
			(*b*)	600	0·0344	0·0115	(0·67)	0·34	Ppt. red
6.	Cellulose (filter paper)	5·090	(*a*)	250	0·0296	—	0·35	—	
			(*b*)	1080	0·0580	—	—	0·69	
7.	Cellulose (No. 5 mercerised)	1·662		1025	0·0227	all	0·00	1·0	
8.	Cellulose (structureless from the xanthate)	2·142	(*a*)	200	0·0358	—	1·04	—	After 180 cc. no aniline acetate reaction
			(*b*)	300	0·0317	—	—	0·9	
9.	Cellulose „	4·753		1560	0·2466	0·1862	0·76	2·4	
10.	Oxycellulose (from No. 7 by ozone)	2·00		660	0·0550	0·0193	1·11	0·66	After 300 cc. no aniline acetate reaction
11.	Lignocellulose (Jute)	1·869	(*a*)	210	0·3291	0·0010	9·4	—	Part soluble was red
			(*b*)	950	0·0357	0·0127	—	0·90	
12.	Lignocellulose (Beech Wood)	2·600	(*a*)	400	0·6909	—	12·68	—	
			(*b*)	660	0·0148	—		0·50	

* (*a*) represents portion of distillate reacting to aniline acetate, (*b*) is the portion subsequently obtained active to the aniline-alcohol reagent.

Discussion of the Results.

(1) These results show that the condensation which produces furfuraldehyde takes place rapidly and is almost completed before the hydroxymethyl derivative begins to distil over. In experiments 5 and 11 for example the

phloroglucides obtained from the first portion of the distillates were practically insoluble in alcohol, consisting entirely of the furfuraldehyde compound. The aniline acetate paper test therefore determines the separation of the two aldehydes and practically no error is made in pentosan estimations if this indicator is used. The possible error is further minimised in the case of bodies such as jute and beech wood which contain a large proportion of pentosan constituent, for the results show that these typical substances give the smallest yields of hydroxymethylfurfuraldehyde (Nos. 11 and 12).

(2) The hydroxyaldehyde is produced at a very slow rate. Its total amount is small, varying between 1 and 2 per cent. in the case of the hexoses and those celluloses which contain little or no pentosan. The probable reason for this is the ready hydrolysis of the hydroxymethylfurfuraldehyde to formic and laevulic acids already mentioned. This property no doubt explains the observations of Fraps [1901]. He found that if the distillates obtained from a number of natural products, food-stuffs, etc. were distilled again a considerable loss of "furfural" was shown. With pure furfuraldehyde this did not occur. The reduction in the amount of pentosan constituent varied from 7 per cent. in the case of oak wood to 22·6 in the case of bran. He considered that this portion could not be regarded as true pentosan, and, in the absence of further knowledge, designated it as the "furaloid" constituent. In all probability however the "furaloid" is a hexose-yielding constituent which gives hydroxymethylfurfuraldehyde and this on a second distillation is largely decomposed.

(3) The constant presence of ω-hydroxymethylfurfuraldehyde in the distillates from cellulose and starch renders determinations of methylpentosan, previously made, open to considerable suspicion. In the only method available, that of Ellet and Tollens [1905], the alcohol-soluble portion of the phloroglucide is calculated as methylpentosan. The work of Fromherz [1906] is interesting in this respect. In order to carry out an examination of the variation in the pentosan and methylpentosan constituents of wood under various alkaline and hydrolytic treatments and anticipating the presence of unknown substances likely to give a precipitate with phloroglucinol he used the method of precipitation with barbituric acid, first suggested by Unger [1902], as a check on the results. Fromherz worked out the application of this reagent to the estimation of methylfurfuraldehyde, finding that combination took place according to the equation:

$$C_6H_6O_2 + C_4H_4O_3N_2 - H_2O = C_{10}H_8O_4N_2.$$

Some of his results with phloroglucinol are as follows:

TABLE IV.

	Furfuraldehyde per cent.	Methyl-furfuraldehyde per cent.
Wood (extracted with alcohol-ether)..	12·68	0·43
„ (6 times treated + NaOH) ..	2·29	0·50
„ (9 times „ „) ..	2·27	0·50

It will be observed that while the pentose constituent represented by the furfuraldehyde obtained is rapidly dissolved by treatment with sodium hydroxide, the methylpentosan remains unchanged in amount. This in itself is exceedingly improbable, but it will further be noticed that the amount of " methylfurfuraldehyde " obtained agrees closely with the amount obtained by ourselves from purified beech wood and shown to consist in all probability of ω-hydroxymethylfurfuraldehyde derived from the cellulose. Unfortunately the barbituric acid method does not permit of a separate estimation of the methylfurfuraldehyde, but calculating by an indirect method Fromherz found that the total of the furfuraldehydes given by phloroglucinol was always higher than that given by barbituric acid, the figures in one case for example being 2·78 and 1·92 per cent. respectively. In a series of experiments we found that in the dilutions commonly obtained in the distillates, ω-hydroxymethylfurfuraldehyde did not give a precipitate at all with barbituric acid. This observation would seem to confirm our view that it is not a case of methyl- but of hydroxymethyl-furfuraldehyde of quite different origin.

(4) Experiments do not throw any light on the remarkable fact that the ketoses give comparatively large yields of hydroxymethylfurfuraldehyde by the action of acids, while the aldoses give little or none. Some of these values calculated as the hydroxy-derivative are given in the following table:

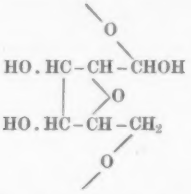
TABLE V.

Yield of ω-hydroxymethylfurfuraldehyde per cent.

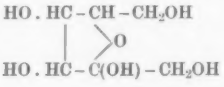
	HBr in chloroform solution[1]	Aqueous oxalic acid[2]	40 % aqueous HCl (sp. g. 1·21)[3]	Aqueous HCl (sp.g. 1·06)[3]
Dextrose ..	2	1	1	1·6
Lævulose ..	15	20 to 25	—	1·1
Cane Sugar	6	—	15 to 20	—
Cellulose ..	20	—	5	0·6

[1] Cross and Bevan.
[2] van Ekenstein and Blanksma.
[3] Dorée and Cunningham.

Although by the action of 40 per cent. aqueous hydrochloric acid cellulose is converted quantitatively into dextrose [Willstätter, 1913], yet with an ethereal solution of the acid it gives a high yield of ω-chloromethylfurfuraldehyde. It has been argued from this that cellulose must have a structure similar to that of laevulose and probably therefore contains a carbonyl grouping in the complex. But on theoretical grounds it seems to us that under anhydrous conditions the transition of laevulose to hydroxymethylfurfuraldehyde is far more likely to take place if the sugar functions in the γ-oxide form (II), and if this is so the cellulose formula (I) given by Green [1906], modified to show a connection between the unit groupings, explains very simply the conversion of cellulose, through laevulose, to ω-hydroxymethylfurfuraldehyde.



I. Cellulose formula (Green) modified for an aggregate of groupings.



II. Laevulose; γ-oxide formula.

Summary.

(1) ω-Hydroxymethylfurfuraldehyde is formed by the action of dilute hydrochloric acid on hexoses, starch and the celluloses. Its amount varies from one to two per cent.

(2) Owing to its slow formation it does not interfere with the accuracy of pentosan estimations made by the Kröber phloroglucinol method, if aniline acetate is used as the indicator.

(3) Its occurrence, however, renders previously made estimations of methylpentosan of doubtful value.

(4) It is probably the unknown substance giving a precipitate with phloroglucinol referred to by previous workers, and its presence explains many of their observations.

REFERENCES.

Dorée (1913), *J. Chem. Soc.* **103**, 1347.
van Ekenstein and Blanksma (1910), *Ber.* **43**, 2355.
Ellet and Tollens (1905), *J. Landwirth.* **53**, 13.
Fischer (1914), *Ber.* **47**, 973.
Fraps (1901), *Amer. Chem. J.* **25**, 201.
Fromherz (1906), *Zeitsch. physiol. Chem.* **50**, 217, 241.
Green (1906), *J. Chem. Soc.* **89**, 811.
Grund (1902), *Zeitsch. physiol. Chem.* **35**, 120.
Kiermayer (1895), *Chem. Zeit.* **19**, 1003.
Kröber (1901), *J. Landwirth.* **49**, 7.
Oshima and Tollens (1901), *Ber.* **34**, 1425.
Reed and Schubert (1914), *J. Amer. Leather Chem. Assoc.* **9**, 131.
Tollens (1902), *Zeitsch. angew. Chem.* **477**, 408.
—— and Goodwin (1904), *Ber.* **37**, 315.
Unger (1902), *Ber.* **35**, 4440.
Willstätter (1913), *Ber.* **46**, 2401.